

More change for British research

The proposed amalgamation of the British research councils (another reorganization, and suspect on that account) deserves a welcome if its management can satisfy some obvious tests.

WHAT will happen next to the British research enterprise? That must have been the question on the minds of members of the Academia Europaea at their second meeting in London this week (see page 651). Mr Kenneth Baker, Secretary of State for Education and Science, is the nearest thing to a minister of science that the British allow themselves (but he must worry about schools and what is taught in them as well). He is also a good minister, one whose avuncular appearance is matched by avuncular inclinations, and who is also the means by which an extra £100 million has been added to the British science budget this year. On Monday, he outlined for a European audience a seamless rationalization of British government policy on research that made the events of the past ten years appear both logical and predetermined. But Mr Baker also made it clear that the process of change has not ended. Like others, he is awaiting with interest the outcome of the next meeting of the Advisory Board for the Research Councils on 19 July at which a decision will be made about the future of the research councils. The motto seems to be that change is always good for them.

The change now in prospect is that foreshadowed by the report from Mr Richard Morris (see *Nature* 18 May, 1989) which, two months ago, came to the conclusion that the simplest way of reconciling frontier disputes between the research councils (the Medical Research Council and the Science and Engineering Research Council have made contentious ground of biotechnology, for example) would be to amalgamate them. Surprisingly, the research councils seem cheerful at the prospect. Only the MRC, pleading its tradition of independence and the distinctiveness of clinical research, threatens recalcitrance. But the only serious objection to this proposal is that it amounts to yet another administrative change in the organization of British civil science after a decade in which there has been more change than people's morale has been able to sustain. Members of the European academy must be aware of the dangers of further upheaval.

The case for biting Mr Morris's bullet is strong, even irresistible; the question for Mr Baker (who would have to approve and then legislate for radical change) is whether the proposed amalgamation of the four research councils concerned with the natural sciences (agriculture and the natural environment as well as those already named) as well as the Economic and Social Research Council would adequately meet the still crying needs in

the mechanism for supporting British research. If lumped together and then endowed with the proposed transfer of up to £70 million a year from the universities' budget (to pay for overhead costs), the system would be spending more than £900 million a year. Mr Baker made a great deal, on Monday, of the need to get rid of paperwork in the application for research grants, but that is not the most serious obstacle now standing between researchers and the bench.

Two issues stand out, of which the most important is what appears to be the incorrigible inclination of the research councils as they are to spend the bulk of their funds on their own establishments. In agriculture, medicine and the natural environment, the explanation is historical — that is largely how their research programmes began in the half-century preceeding the upheavals of the early 1960s. The SERC, for the same or apparently equally good reasons (the economical provision of central services for its university constituents), has acquired the large Rutherford-Appleton Laboratory, two national observatories (one in Scotland) and a synchrotron radiation laboratory. There is nothing wrong with these establishments, which command the loyalty of those who work in them and which collectively are the source of much excellent work, but administratively they are a fixed cost and thus an obstacle to flexibility. Thus one crucial question to ask about the new super-research council is whether it would be managed in a decisively different way. Mr Baker should listen hard, in the next few weeks, for noises to that effect.

The other criterion by which the proposal for amalgamation should be judged is that of whether it would assist the international collaboration whose need is ever more urgent. Mr Baker said all the right things on Monday about the difficulties of small countries such as Britain when faced with the temptation to "do everything at once". The imaginative way out of this cleft stick would be to give the amalgamated council an international dimension, allowing it to make deals off its own bat (and not through the government that supports it) with like-minded organizations elsewhere, especially in Europe. In that respect, of course, Mr Baker will not be entirely dependent on the Advisory Board for advice. There is much that he could do himself to nudge the new pattern in a beneficent direction. But on past form, there will have to be a lot of nudging.

□



British backsliders

This week's summit meeting of European politicians at Madrid argues for a redefinition of Europe.

THE British are a perpetual source of perplexity to other Europeans, notably those living on the mainland. Do they belong, or do they not? This question was made clamant again this week at the Madrid summit at which eleven heads of government of the members of the European Community (EC) sought to persuade the twelfth — Mrs Margaret Thatcher, the British prime minister — that the British must sooner rather than later decide where they stand on Europe. They will probably have to wait some time before they get a clear answer.

The British themselves, by their long-standing ambivalence towards the mainland, are chiefly to blame. In three decades, there have been three upheavals of opinion about the EC (the rejected application for membership in 1962, the successful application a decade later and the succeeding referendum when British voters were asked whether they wished to continue to belong). Paradoxically, the present government seemed, at its election ten years ago, an enthusiast for membership. In 1984 it signed the amendment to the Treaty of Rome known as the European Single Act, the legislation that will make Europe into a true common market at the end of 1992. Since then Mrs Thatcher, at least, seems to have gone sour on the European project. Her infamous speech at Bruges last year showed that. What has gone wrong?

It is too simple, but probably not irrelevant, to say that Mrs Thatcher and M. Jacques Delors, the president of the European Commission, have little liking for each other. It is also relevant that Britain, now economically in one of its recurrent bad patches, can hardly be expected to act confidently. But what now also seems plain is that Mrs Thatcher, having signed the Single Act, feels impelled in unwelcome directions by some of its implications. That certainly applies to the row at Madrid this week about the European Monetary System, a scheme for permanently linking together the different European currencies with which most EC members have lived cheerfully (and, some say, profitably) for the past decade.

Now M. Delors has mapped out a three-stage programme in which membership of the monetary system would be followed by the creation of a European Central Bank to regulate the monetary system and, eventually, by a common European currency. Mrs Thatcher protests that these are steps towards some distant United States of Europe to which Britain is not committed. But is that so? How could a common market function efficiently if European traders were not able to tell in advance what they would get for what they sold until Europe's banks had converted payments from one currency to another (and taken a cut in the process). The notion that there should be a single currency is a simple consequence of the Single Act. If those who signed that act failed to anticipate

that a common monetary policy would be required, who but themselves can they blame?

That is half the story. The more charitable explanation of Mrs Thatcher's discontent with the EC is that it has been put together backwards. The objective of the Treaty of Rome was openly to capture in Europe the economic benefits apparent in great federations such as that of the United States. In the 1950s, it was cheerfully supposed that the task could be accomplished in isolation, without affecting political and administrative arrangements in member states.

What now emerges is what should have been predicted — that economic integration entails other kinds of integration which, nevertheless, need not amount to political integration. The official British position now is that only developments towards the economic goal are allowable — and the definition is being tightly drawn. (Mrs Linda Chalker, Minister of State at the British Foreign Office, said the other day that she was "watching Brussels like a hawk" to make sure that the Commission did not exceed its powers in education, apparently careless of the benefits that might flow from regarding European universities as a common resource.) Other European governments are more sanguine. Mrs Thatcher (who may have misjudged her electorate on the European issue) and her opponents would find future meetings with each other more fruitful if they began at the other end, listing the forms of collaboration outside the strictly economic field that would be mutually beneficial and those implied, but which are not explicit, in the Treaty of Rome and the Single Act. Then, at least, they (and the rest of us) would know the bill of goods. □

A most rotten fate

Sakharov, an angry man, must square his own emotional circle before squaring the Soviet Union's.

ACADEMICIAN Andrei Sakharov, in London last week (see page 651), is both genius and saint. He is also a very angry man, and for good reason. He was illegally exiled to Gorkii by a previous regime and, while there, accused by a shameful number of his colleagues in science of something akin to treason. It is lucky for all concerned, and not just Sakharov, that he was not killed.

But, like the visions of other genius-saints (there have not been many), Sakharov's visions are distant visions. (Augustine's *The City of God* refers.) He has the cheek to believe not merely that there should already be real democracy in the Soviet Union, but that its ingenious people could solve its impossible economic problems in a day or so. He is right, of course: they could. But they will not. The best hope is that it will take a few more years. His worry, like that of the rest of us, is that it will never happen. Then, of course, we should all be dead. So why not, like a card-player who has figured out that it is possible to win only if the queen of spades is on the left, give fate the benefit of the doubt? □

More grief and despair for Chinese students

- US protest groups form
- Academic exchanges end

Washington, Boston and San Francisco

Almost one month after unarmed Chinese students and workers were shot down by the People's Liberation Army in Tiananmen Square, the "round-up of counter-revolutionaries" continues. Last week a list of seven wanted intellectuals was circulated by the Central Committee of the Communist Party. Police raided campuses of Beijing College, seizing 15 students and teachers. More than 2,000 people are known to have been arrested and 27 executed.

The names of those killed in Beijing on June 3 and 4 or arrested afterwards are slowly being compiled by human rights organizations. Among those reported dead are Hao Zi Jing, a research assistant in the Chinese Academy of Sciences; Sun Hun, a student in the chemistry department of Beijing University; Xiao Bo, a lecturer in the chemistry department of Beijing University; Yan Wen, a student of statistics at Beijing University; Wang Weiping of Beijing Medical University; Zhong Qing, of the optical instrument department at Qinghua University; Wu Xiandong, a student at Beijing Electronic Instrument Work College.

Those accused of "spreading rumours", "shouting reactionary slogans" or of carrying out acts of "vandalism" are being arrested. Arrests reported by the Asia Watch human rights group include Liu Gang, a physics graduate from Beijing University, one of the "21 Most Wanted" student leaders; Zhou Fengsuo, a physics student at Qinghua University; Ma Hongliang, a student of the Xian Institute of Metallurgy; and Guo Haifeng, secretary-general of the United Association of Beijing Universities.

Chinese students in the United States are still stunned by events, their grief intensified by eye-witness accounts of the killings from a few fellow students who have managed to return to the United States and by gruesome pictures of the Tiananmen Square carnage. As the initial feelings of horror and outrage disappear they are being replaced by frustration, disillusionment and despair. "All our dreams were broken by the massacre," said one student. "All of us who had come here to study, to try and build a better country — now everything has changed. Now you have to separate your feelings: you love your country, but the government is criminal. Chinese intellectuals are now in a very dangerous situation." The first reaction was to try to help those in China.

Students at the University of California at Berkeley, for example, raised \$7,500 to help the injured and the families of those killed. But as yet they have found no means to send the money to China.

Hundreds of students have tried to hit back at the Chinese government campaign of misinformation by joining in a campaign to jam the phone lines of a special police switchboard set up for informers in

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

The Tiananmen Square "Goddess of Democracy" (above) was destroyed by the tanks of the People's Liberation Army, but a replica now stands in a small park outside the Chinese Embassy in Washington, DC.

Beijing. When the students did get through and were asked to identify a criminal, they responded: "Deng Xiaoping". Individual actions are now gradually giving way to a more organized response. At Berkeley, where there are more than 400 Chinese students, a coordination committee has been set up to try to lobby Congressmen and to try to get the truth through to China. A spokesman said that they knew some fax messages were still being received in China.

In Boston, students at the many local universities have set up a China Information Center. Yuan Liu, a Brandeis University student said the centre's aims were three fold: "to use communication as a weapon; to break Chinese news control by whatever means we can; and to compile names of those arrested and killed as a tool to pressure the government".

Yuan explained that Chinese students are in a dangerous position. "If you're

involved in the movement in any way you are taking a risk. It is certain that you would be arrested if you returned to China. Even if you were not really politically involved here, that doesn't matter." Simply by being in the West, Chinese students and academics may be considered suspect.

Many Chinese students and academics are reluctant to join organized protest groups, fearing that they are being observed constantly. Demonstrations outside the Chinese Embassy are said to have been taped. And while students have welcomed the US government's offer to extend their visas, many fear that they will face extra difficulties when they do decide to return home. At present, it is necessary to declare oneself unwilling to return to China in order to obtain the extension.

Nationwide organizations of Chinese students look set to emerge in the near future. John Cwapisz, an attorney at the Washington-based Center for Peace and Freedom said that 60 groups have come together under the China Democracy Coalition with a range of ideas for action, including organizing a benefit concert with top pop stars to raise money, and a plan to raise a monument to those who died in Beijing. The first official memorial is likely to be the naming of the park in front of the Chinese Embassy in Washington as the Tiananmen Square Memorial Park. A bill has already passed the Senate and will go before the House this week.

All cultural and academic exchanges between the United States and China are grinding to a halt. The Institute for International Education administers several exchange programmes, most of which have been postponed. Those of the National Academy of Sciences have been postponed. The National Science Foundation (NSF) has suspended all staff travel to China and is advising grantees not to travel there. The intergovernmental Cooperative Agreement on Science and Technology, which is the umbrella for several US-China collaborative programmes, reaches the end of its five-year lifetime in October and is unlikely now to be renewed.

Universities and agencies are trying to find out exactly what the needs are of the 30,000 Chinese students and 10,000 academics in the United States. NSF says it will consider requests for financial support for grantees who opt to extend their visas. Several hundred academics may be eligible to apply but NSF does not know yet exactly where the money will come from.

One plan under discussion is for NSF to transfer money (some \$3-5 million) budgeted for a cooperative programme with China into a fund that could benefit Chinese students here who are afraid to return.

**Christine McGourty,
Seth Shulman and Alun Anderson**

Associated Press

The flatworm's turn

Washington

THE question of which person's genes will be mapped and sequenced by the US human genome initiative is still open, but under the genome programme of the US National Institutes of Health (NIH), the humble nematode may be the first higher organism to have its genes ordered into a physical map. At its second meeting last week, NIH's advisory committee on the human genome project unanimously agreed to endorse a joint effort by US and British researchers to finish the nematode gene map.

It has always been clear that the genome effort will not provide the sequence of one individual's genes, but rather an amalgam of human DNA from various sources. But mapping and sequencing human genes will be only a part of the genome initiative: further studies of the genomes of such well-studied 'model organisms' as the *Escherichia coli* bacterium, the fruit fly *Drosophila*, the nematode and the mouse have been discussed as stepping-stones towards the human genome.

James Watson, who divides his time as director of both the Cold Spring Harbor Laboratory and the NIH Office of Human Genome Activities, brought up the idea of putting NIH's initial efforts into sequencing the nematode genome at the advisory committee meeting. He says researchers at the British Medical Research Council's (MRC) Laboratory of Molecular Biology at Cambridge and at Washington University Medical School in St Louis, Missouri, have broken down the genome of the nematode into 200 sets of overlapping DNA fragments, or 'contigs'.

By bridging breaks between contigs

using the yeast artificial chromosome technique developed at Washington University (see *Nature* 335, 184; 1988), Watson believes the team can whittle the nematode genome down to 100 contigs in roughly a year. Putting the contigs in their correct order will provide a physical map of the nematode, which he believes can then be sequenced by a team of 50 technicians within six years.

Watson estimates that a three-year, \$600,000 grant would allow the US-British collaboration to finish the physical map and start sequencing. At 100 million base pairs of DNA, the nematode genome is roughly the size of an average human chromosome. The nematode's biology is so well understood — it is known to have exactly 958 cells, and each cell division during development has been completely described — Watson says "the worm people may lead the way" in genome research.

But Congress is not likely to smile on a proposal to spend part of the \$100 million the Bush administration has earmarked for the genome initiative next year on a worm, especially if part of it will go to research outside the United States. The NIH genome advisory committee, chaired by Norton Zinder of Rockefeller University, must report to Congress next spring on how the genome project should proceed. In the meantime, MRC's Michael Kemp says he has a proposal from the British group on his desk, and Washington University's Robert Waterston says his group is "doing its homework" to submit a grant application once NIH issues its request for applications for 1990 genome programme grants next month.

Carol Ezzell

HUMAN GENOME

Sequencing by committee

Tokyo

WHATEVER happened to the human genome project in Japan? A couple of years ago, there were fears in the United States that if a project were not started there immediately, Japan would steal the lead. But while the United States and Europe have long since established organizations to sequence the human genome, Japan's efforts have hardly got beyond the committee report stage.

Last month a subcommittee of the Science Council of Japan, a non-government body elected directly by academics, issued a report recommending a greatly expanded effort. Two subcommittees of the Ministry of Education, Science and Culture also recently submitted similar recommendations to the ministry's own science council. And last year the Science and Technology Agency (STA) issued a

vaguely-worded call for a project but gave no indication of the direction it should take (*Nature* 334, 5; 7 July 1988).

But apart from a small-scale effort at STA's institute of physical and chemical research (RIKEN) to develop automatic DNA sequencing machines, a project started many years ago by Professor Akiyoshi Wada of Tokyo University, there is no project under way in Japan.

Kenichi Matsubara of the Institute of Molecular and Cellular Biology of Osaka University, head of one of the education ministry's subcommittees, says that, even if the ministry's science council accepts the subcommittee's recommendations when it meets to discuss them next month, it will be at least two years before government funds will be available to support a project.

Meanwhile, with the council's approval, Matsubara says the subcommittee

Lyons laboratory monkeys recaptured

Paris

TWENTY-EIGHT of the monkeys stolen in a raid on laboratories in Lyons earlier this month have been found in a private house at Toulon (see *Nature* 339, 407; 1989). Altogether, 38 monkeys and some marsupials, as well as dogs and cats, were removed in a raid by an animal rights group.

Five people were arrested in connection with the discovered animals and two have been retained for further questioning. The monkeys are said by a researcher at one of the laboratories concerned, Unit 97 of the institutes for health and medical research (INSERM), to be "in very good condition".

Police have so far not returned the monkeys to their owners for fear of provoking public protest. Since the raid, the French press has published several articles showing gruesome pictures of laboratory animals, including photographs taken during the Lyons raid.

Last Friday, animal rights campaigner Brigitte Bardot broadcast a powerful anti-vivisection documentary on French television, with a well-known cancer researcher (and former health minister), Leon Schwartzenberg, speaking against the use of animals for experiments. The broadcast gave no opportunity to biomedical researchers to defend their case and will not help their recent efforts to put animal experimentation into perspective (see *Nature* 339, 573; 1989).

Peter Coles

hopes to launch a "small rocket" using emergency funds that the ministry sets aside for research on earthquakes and the like. The funds will be used to organize more committees of university researchers and to cope with demands for genome information from overseas by, for example, improving computer programming facilities.

The report from the Science Council of Japan calls for the establishment of an organization to coordinate a joint research effort by various government agencies and ministries: this is not an easy task in Japan because of inter-ministerial rivalries. Nevertheless, Matsubara hopes that by drawing together university researchers with 'emergency' funds from the Ministry of Education, an 'invisible committee' will be established during the next two years that could coordinate an inter-agency project.

Matsubara is Japan's representative of the Human Genome Organization (HUGO), established last year to coordinate worldwide efforts on the project; he has been trying to raise funds in Japan to support HUGO, so far without much success. As well as calling on private companies, he has approached organizers of Japan's Human Frontier Science Program.

David Swinbanks

AUSTRIAN SCIENCE POLICY

Hankering after Europe

Vienna

As an indication of its desire for closer contacts with the European Community, Austria gave an enthusiastic reception to the Eureka conference of Industry Ministers in Vienna last week (some of the meeting's decisions are covered on page 652). There is "no alternative" to joining the EC if Austria wants to improve its science and technology base, says new Austrian Science Minister Erhard Busek. Most important, he says, would be gaining access to EC science programmes.

Even if it applied soon for EC membership, Austria could not hope to be accepted before 1992, when the single market is supposed to come into effect.



Busek — increasing Austrian competitiveness.

Whether to apply or not is at the top of the political agenda, but the question may not be settled for many months. Busek jokes that "Hungary may be a member sooner than Austria". In the meantime, this small country has other pressing needs in the areas of education and science.

Busek inherited a difficult task from his predecessor, biochemist Hans Tuppy. With less than 18 months remaining in the term of the current coalition government, Busek has to soothe interest groups in the universities and industry while trying to drum up more R&D investment from government and industry sources. Although Tuppy achieved a significant increase in the Science Ministry budget for 1989, pressure from critics, especially those in universities, helped to force him from office.

Busek's first priority is to negotiate another budget increase for 1990. The government promised in 1987 that Austria would increase total spending on research investment from public and private sources equal to 1.5 per cent of the gross domestic product. Although far less than

the 2.5 to 3.0 per cent invested by West Germany, Japan and the United States, this would nevertheless be a substantial increase over the current level of 1.3 per cent investment (roughly 21,900 million Austrian schillings, or US\$1,564 million, in 1989).

It seems likely that the government will have trouble keeping its promise, although Busek remains hopeful. Whatever the outcome, Busek intends to pursue a strategy of filling niches in order to increase Austrian competitiveness. He hopes to attract more research institutes like the Institute for Molecular Pathology (IMP), founded jointly in Vienna last year by Boehringer Ingelheim and Genentech with Austrian state support.

More recently, a new institute for laser research has been announced at the Mountain University of Leoben, and Siemens and Philips have expanded their efforts in microelectronics research in Austria.

One niche in which Austria does not necessarily belong, said Busek, is space travel. Austria has agreed to pay the Soviet Union 150 million schillings (US \$10.7 million) to put an Austrian astronaut on the Soviet MIR space station in late 1991 or early 1992. Busek is sceptical about the scientific merit of this arrangement, but he says that it was mostly a "political decision with which we have to live".

Busek supports the current initiative of the Austrian Academy of Sciences to establish guidelines for genetic engineering as a way to secure the future of biotechnology research in Austria. Though adherence will be voluntary, Busek sees the guidelines as a sign of responsibility on the part of researchers. In addition, the Ministry has commissioned a report, due next February, on how best to regulate genetic engineering both in research laboratories and in production. Karl Schlögl, who chairs the Academy committee responsible for the new guidelines, sees his work as pre-empting the actions of a parliament that might all too easily decide to ban genetic engineering. Schlögl hopes to present the committee's recommended guidelines by the autumn of this year.

Busek stands by the long-term goal of the research community that 3 per cent of the Austrian gross domestic product be spent on research and development by the mid-1990s. Busek hopes this goal is realistic, but recognizes that priorities will have to change tremendously for it to be achieved. "It would be better to put our billions into science and technology rather than the Autobahn. We might even get some support on this from the environmentalists."

Steven Dickman

SPACE STATION

Japan joins in at the last minute

Tokyo

JAPAN will at last be able to join Europe, Canada and the United States in the construction phase of the US Space Station. A motion approving the inter-governmental agreement between the United States and Japan on the station was passed last week by the Diet.

The motion has been stalled in the Diet for months because of the opposition's boycott of Diet deliberations to protest the Recruit scandal, the discovery that many key members of the ruling Liberal Democratic Party made huge profits through the buying and selling of shares. And there were fears that the motion would not pass before the summer recess of the Diet (*Nature* 339, 495; 1989).

But despite opposition objections to possible military uses of the space station, the motion finally passed on the last day of Diet deliberations. David Swinbanks

INSAT

Indian hopes and satellite dented

New Delhi

THE luckless Indian space programme faltered again when its newest communications satellite, Insat-1D, was severely damaged in an accident at Cape Canaveral, Florida, barely a week before the scheduled launch on 29 June. The setback jeopardizes India's \$2,000 million domestic satellite communications network: only Insat-1B is now fully operational, but it is reaching the end of its six-year design lifetime. Insat-1A failed within two weeks of its launch, and Insat-1C is operating at less than half capacity after a power supply failure.

The \$53-million dollar satellite, made by Ford Aerospace in the United States, was hit by a falling 45-kg hook when a cable on a service crane broke. At best repairs will take many weeks. The launch, using a Delta rocket, was to have been the first commercial operation by McDonnell Douglas, who leased facilities at Cape Canaveral from the US Air Force.

The accident has dashed hopes of replacing Insat-1B during its lifetime and threatens not only India's 272 television stations but also the politicians who hope to use their services in the November general elections.

The only hope for the Indian Department of Space seems to be somehow to extend the life of Insat-1B beyond September, because none of Intelsat's three Indian Ocean satellites has any spare capacity. Intelsat may try to help India by securing one of the yet to be launched Arabsat satellites, but this will not solve the short-term crisis as no suitable launcher is available for another 18 months. K. S. Jayaraman

World Bank's hint of green

Washington

A \$20 million World Bank forestry loan to Sri Lanka, approved on 15 June, is being welcomed by environmentalists as an indication that the Bank is finally taking environmental issues seriously.

Pressure from local and international environmental groups resulted in last-minute renegotiation of the terms of the loan to incorporate stringent environmental safeguards, including suspension of logging in natural forest areas until environmental impact assessments are carried out.

But despite a commitment to giving environmental issues higher priority, announced in a 1987 speech by World Bank president Barber Conable, critics say little has changed. There is now an environment department and four regional units, but the new department is said to have too few technical experts and to have little influence over powerful regional directors.

The Environmental Defense Fund (EDF), long a stern critic of the Bank's environmental record, is due to give evidence before the Senate on 10 July, and although some positive changes will be reported, its criticisms will be modified only slightly from those of previous years.

In evidence to the House subcommittee on foreign operations, Bruce Rich, a senior attorney with the EDF, said that "for every case where there appear to be promising changes we can identify two or three ecological and social debacles of equal or greater scale where the Bank refuses to act even when apprised of the facts".

The director of the Natural Resources

Defense Council, Thomas Stoel, said that the Bank's environmental impact assessment was unsystematic and did not meet "even the minimum accepted international standards". He also said the Bank should involve non-governmental organizations and the general public from donor and borrower countries in the design of projects, and he criticized its "strict secrecy".

Conable said last week that the Bank's new emphasis on environmental reform "had not been easy". Some developing countries resist environmental programmes because they are seen as being "foisted upon them" by industrialized countries. They perceive that "the advanced countries have found yet another excuse to impede the development of poor countries and to encroach upon national sovereignty in a modern-day version of colonialism. He pointed out that more than 100 projects with significant environmental components will have been approved by the end of this year. Over the past two years almost \$500 million has been lent for projects that protect or develop forests, and that sum will be more than doubled in the next three years. He also said the Bank plans to lend \$1,330 million for environmental projects over the same period.

Jeremy Warford, an attorney in the Bank's environment department, says that environmental reform within the Bank has proceeded at a "tremendous pace". To criticize individual projects is misleading, he says, since the principal thrust of the Bank's environmental policy is to integrate environmental issues into loans to governments for macroeconomic change. By 1989 a comprehensive environmental report on each borrowing country will have been prepared and will be fed into the policy-making process.

The Senate foreign operations appropriations subcommittee, which has for more than six years kept up pressure on the Bank to introduce environmental reforms, won another victory last week when the treasury secretary Nicholas Brady agreed to include 'debt-for-nature' provisions into the so-called "Brady Plan" for the reduction of debt in developing countries. Under these provisions, a portion of a nation's foreign debt is purchased at a discount in exchange for a commitment by the debtor nation to environmental initiatives. Though the details of the plan are not yet clear, it is a significant change from the policies of previous administrations which have held that all of the debt should be repaid on market terms.

Christine McGourty

■ The economic value of non-timber products of tropical forests is rarely taken into account — see the Commentary on page 655.

Queensland's space base threatened

Sydney

PLANS for a commercial spaceport at Cape York, in the far north of Queensland, may disintegrate now that one of the two consortia formed to develop the proposal has dropped out of the running, claiming that demand for the facility will be insufficient to make the A\$350 million project commercially feasible until the late 1990s.

A rival consortium, the Cape York Space Agency (CYSA), now intends to pursue the project, but concerns over international technology transfer issues, as well as pressure from environmental and aboriginal groups, also threaten the success of the proposed space base.

The first launch, planned by CYSA for late 1992, will use Soviet equipment, but this may prevent NATO member countries from using the spaceport because of regulations enacted by Cocom, the NATO Coordinating Committee on East-West trade policy, aimed at keeping US technology out of Soviet hands. Australian federal officials met their US counterparts in Washington last week, and are expected to meet again on 16 July, to clarify security controls and the extent of likely technology transfer resulting from use of the spaceport. With agreement between Australia and the United States, United Technologies, the US aerospace company, could be an equal joint partner with CYSA and the Japan Cape York Spaceport group.

But there are also problems on the home front. After the formal submission of plans by CYSA, expected in two months, the government will set guidelines for an environmental impact study. Objections are expected from environmental and aboriginal organizations.

Queensland's Minister for Industry and Technology, Rob Burbidge, has said that a special Cape York enterprise zone will be set up, allowing various tax advantages and freight and electricity concessions, but part of the site is on the Register of the National Estate, and is an area of national significance, according to Liz Bourne, coordinator of the Queensland Conservation Council.

Stephen Williams of CYSA says that neither trade nor environmental issues will force abandonment of the project. CYSA will rely on the federal government to solve the technology transfer problems, and will abide by any environmental restraints that are set by the Queensland government. "We still think it's a commercially viable project", says Williams.

Tania Ewing

EUROPEAN COMMISSION

Call for European version of EPA

Paris

THE success of ecology parties in last week's European Parliament elections has given new life to European Community (EC) proposals to set up an agency to monitor pollution. A revived initiative was announced in Brussels last week by the EC Environment Commissioner, Mr Carlo Ripa di Meana. The aim of the agency would be to gather comparable data from the twelve member states on air, water and soil pollution. Britain has voiced reservations on the grounds that such an agency could interfere with existing national organizations, but some EC members do not have extensive monitoring structures and, at present, measures taken by other states sometimes use differing criteria, making Europe-wide controls difficult.

Peter Coles

SAKHAROV

Gorbachev's candid friend

London

ACADEMICIAN Andrei Sakharov last week warned the West against increasing economic and scientific aid to the Soviet Union. In the absence of radical reforms of the Soviet system, he said, such assistance could serve only to prop up an ailing system and delay the advent of democracy. It would be "pouring water into the sand" to increase assistance at this time.

Sakharov, in Britain to receive honorary degrees from the universities of Sussex and Oxford, delivered his warnings at the Royal Institute of International Affairs. He described the present situation of the Soviet Union as one of chronic shortage, a bankrupt economy and a national debt that is, *per capita*, higher than that of any other country in the world.

The current "economic reforms", Sakharov maintained, are not working, since the officials in charge of implementing them have no real authority or independence. At the same time, there is an increase of political awareness, which culminated in the run-up to the elections to the Congress of People's Deputies, and a growing ethnic consciousness of the non-Russian peoples of the Soviet Union.

"Our state is the last empire in the world", Sakharov said, adding that if the ethnic problems are not solved in a "constitutional, rational way", civil war or a right-wing coup could well ensue.

The ethnic problem, Sakharov said, overlaps with that of the environment, since the central planners in Moscow have imposed disastrous decisions in conditions of which they had no knowledge. He cited the notorious case of Uzbekistan, where a cotton monoculture has been forced on the population.

The Uzbeks, Sakharov said, are compelled to grow cotton even on their private allotments. Workers in the cotton-fields — for the most part, students and children who lose up to two months study each year — are poisoned with pesticides and defoliants so that the majority of national servicemen from Uzbekistan are unfit for the army.

To deal with this and similar problems, Sakharov said, radical reforms are needed. The problems of the Uzbekistan cotton fields have, in fact, been well-known to Western observers for years. Sakharov, however, spoke of them with all the intensity of one newly aware of the situation. But, as he explained, one of the most important achievements of the Congress of People's Deputies was to make delegates, and through them the Soviet public, aware of just how bad the economic and political situation in the Soviet Union really is.

Under such circumstances, Sakharov was reluctant to give a prognosis. The

Soviet Union, he said, had reached a turning point, and there was no certainty which way it would go — towards hard-line conservatism, or towards greater reform. His view of Mikhail Gorbachev seemed ambivalent; he praised the Soviet leader's current stance, yet, at the same time, emphasized that he had come to power as a result of agreements within the Central Committee of which the Soviet public still know very little.

Gorbachev, he said, had changed the constitution in order to reach a position of power without the necessity of election. Sakharov praised him for *perestroika*, but was plainly worried that Gorbachev might find it simpler to exercise the power in his hands than to deal with the problems around him. But Sakharov acknowledged that he is the only leader in sight.

Vera Rich

AIDS

US forecast too low?

Washington

THE US Centers for Disease Control (CDC) underestimated the number of AIDS cases the United States can expect by the end of 1991 by as much as one-third, according to a report released this week by the US General Accounting Office (GAO). The GAO says the 195,000–320,000 cases projected by CDC is too low, and that a figure of between 300,000 and 485,000 is closer to the mark.

The GAO scrutinized 13 different forecasts of the AIDS epidemic and found serious errors in the underlying data on which the projections were based. Delays in the reporting of cases to CDC by local health departments, and the exclusion of many AIDS cases before the definition of the disease was broadened in 1987, were cited as the principal problems. The agency also found that AIDS cases arising from heterosexual contact were less likely to be accurately reported, and that in many forecasts heterosexual intercourse was simply assumed not to be the cause of AIDS infection if other risk factors, such as drug use, were also present.

To come up with better estimates of the spread of HIV infection, GAO recommends that the US Secretary for Health and Human Services, Louis W. Sullivan, should authorize further studies into the biases of AIDS case reporting, and examine whether CDC needs more resources to do the job. GAO also supports a nationwide assessment of risk behaviours, such as the survey of sex practices proposed by the National Institutes of Health.

Carol Ezzell

EUROPEAN ACADEMY

Future still vague for new venture

London

THE need for international collaboration in the research effort to understand global environmental problems was stressed by Kenneth Baker, the British Secretary of State for Education and Science, opening the first plenary session of the Academia Europaea (AE) in London on Monday.

On the pretext that an overview of British science policy would be to the general enlightenment of a body comprising over 600 senior academics representing the Academies of Arts and Sciences of 20 countries, Baker took the opportunity to re-iterate the often controversial science policy objectives espoused by the 10-year-old government of Prime Minister Margaret Thatcher.

The AE itself was conceived as a supranational version of the Royal Society which, with the British Academy, has so far been the focus of the Academia's initiatives. The idea of an Academy covering science and humanities in Europe, was developed by a group of scholars meeting at the Royal Society in 1985. But the AE came into existence only in September last year when the 100 or so invited 'Foundation Members' met at Cambridge.

Renewable resources and human origins were on the agenda at the London meeting this week, but arrangements for the next meeting, in Strasbourg next year, are not yet finalized. Neither, it seems, are the detailed objectives of the AE, except that it will concern itself with research issues of relevance to the entire continent "from the Urals to the Bosphorus", in the words of AE President, Sir Arnold Burgen of the University of Cambridge. Higher education and East-West relations will be high on the list of priorities.

Henry Gee

THINK TANK

Academy to leave Berlin

Munich

THE two-year-old Academy of Science and Technology in Berlin will definitely have to leave the city, after a resolution to close it was introduced last week in the West Berlin Senate. The academy was founded in 1987 by a Christian Democratic government as a think tank for issues concerning technology and society, but was derided by opposition Greens and Social Democrats as an "old men's debating club". Meanwhile, the Land of Hesse has offered to pay the DM8 million operating costs of the academy should it wish to move there, probably to the Frankfurt area.

Academy spokesman Eberhard Vogt expects the closure resolution to pass this September and says that the moving date of January 1990 offered by Hesse is "realistic".

Steven Dickman

Faster connections for 1996

Washington

THE apparent lack of urgency with which the United States is taking commercial advantage of supercomputers and high-speed telecommunications, two areas where the country has an undoubted technological lead, was the focus of two congressional hearings last week.

The House of Representatives committee on Science, Space and Technology bemoaned the failure of US industry to purchase supercomputers as eagerly as their Japanese competitors, even though one American company, Cray, holds three-quarters of the world market. And at a hearing of the Senate committee on Commerce, Science and Transportation, witnesses and senators were almost unanimously enthusiastic in their endorsement of a proposal to build a nationwide high-speed computer network by 1996.

The recent withdrawal of Control Data Corporation from the supercomputer manufacturing business has prompted doubts about the prospects for Cray's success against emerging Japanese competitors such as NEC, Hitachi and Fujitsu. Although the worldwide supercomputer market, at about \$1,000 million, is not by itself a large part of the US economy, it was portrayed in both hearings as essential to research and development in technological industry as a whole. John

five years to build a 3-gigabit-a-second computer network connecting universities, government laboratories, industry and even schools.

The aim is to encourage widespread innovation and use of computer and communications technology by making it available to all. The eventual goal is for the network to be run as a commercial enterprise, but the introduction of the bill amounts to a recognition that market forces alone do not provide enough pressure for business interests to undertake the long-term investment needed.

The National Science Foundation (NSF) already operates, through an industrial consortium called MERIT, a national computer network operating at 1.5 megabits a second. Since its inauguration last year (*Nature* 334, 374; 1988), traffic on NSFNET has increased by about 30 per cent a month, and an enhancement to 45 Mbits per second will be needed next year if the network is not to be overloaded.

The apparently insatiable demand for transmission capacity gives Gore hope that the proposed 3-Gbit network will have crossed the threshold to feasible commercialization almost as soon as it is built. In Gore's bill, the necessary research and development will be handled by the Defense Advanced Research Projects Agency (DARPA), but the coordination

Europeans to design chips for everything?

Vienna

EUROPEAN microelectronics collaboration on an unprecedented scale was agreed last week by five nations and the European Community (EC) Commission as well as three electronics companies. The JESSI (Joint European Submicron Silicon) project will invest as much as 3,800 million ECU (some \$4,000 million) over eight years on the eventual development of a 64-megabit silicon memory chip. But around one-third of the proposed budget will be devoted to designing chips for special applications, and nearly one-quarter will go to research on design and integration methods.

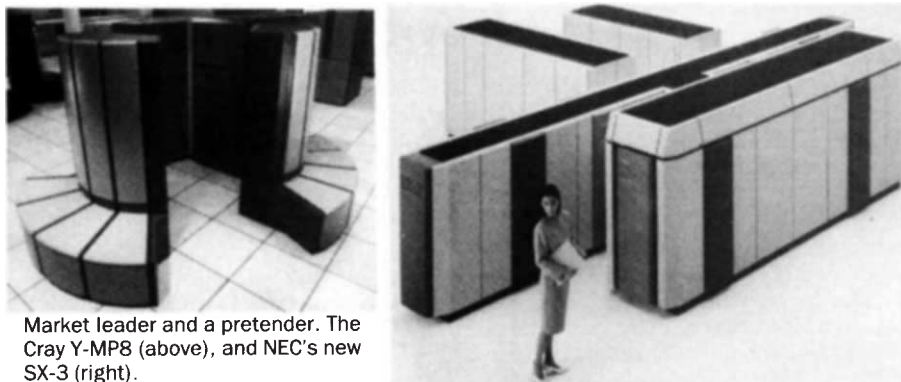
JESSI was announced by the West German government, one of its sponsors, in February (see *Nature* 337, 682; 1989). France, Great Britain, Italy and the Netherlands are also participating, as are electronics companies Siemens, Philips and SGS-Thomson. British involvement in JESSI will focus primarily on the development of application-specific integrated circuits or ASICs. These so-called "designer chips" represent a large share of the market.

Provisional approval of JESSI under the 19-country Eureka programme, pending settlement of a few minor details, was announced in Vienna last week at a meeting of industry ministers. By being brought under the Eureka umbrella, JESSI can be harmonized with related European initiatives, which might have made the EC Commission more comfortable about putting money into it. The EC contribution is expected to be 25 per cent of the 550 million ECU start-up costs. A decision will be made later about EC participation beyond an initial 18-month period. Half of the funding will be provided by the companies involved, and the remaining 25 per cent will come from individual national governments.

The rousing start for JESSI reflects European fears of being shut out of the market for chips by the Japanese, who produce a large percentage of the chips available on the world market. Whether JESSI will be open to participation by US companies like IBM remains an open question; JESSI leaders have made this dependent on US companies allowing European participation in Sematech, a US microelectronics initiative. Steven Dickman

dedented method of funding will undoubtedly run into trouble.

Supercomputers and networks are seen as a touchstone of US technological prowess, and Gore's bill has wide bipartisan support. The hope is that Congress will give serious consideration to an innovative method of financing the network; such an innovation could be as important to the United States as the substance of the bill itself. David Lindley



Market leader and a pretender. The Cray Y-MP8 (above), and NEC's new SX-3 (right).

Rollwagen, chairman of Cray Research, observed that his company had sold supercomputers to 16 car manufacturers, of which only three were US companies.

Asked why it mattered whether industries bought US or Japanese supercomputers, as long as they used them, Rollwagen argued that his company thrived and developed new products by listening to the needs of its customers: if his company failed, it would be because industry generally failed to understand what supercomputers could do.

With signs of another US technological lead slipping away, Senator Albert Gore (Democrat-Tennessee) introduced earlier this year the National High-Performance Computer Technology Act, one section of which would set aside \$1,750 million over

and management of the project will be overseen by the NSF. The Department of Commerce, the National Security Agency and the National Institutes of Science and Technology are also involved.

The traditional way of financing such a plan would be to estimate how much each agency needs and put the contributions into the federal budget at the appropriate places, but this would not guarantee that all parts of the programme would get the money they need. To get around this, the bill gives all of the money to the Office of Science and Technology Policy (OSTP), which would in turn allocate it as needed to the various agencies. Because the bill gives administrative powers over other agencies to a body which has so far acted only in an advisory capacity, this unprece-

GENETIC ENGINEERING

Danish law to be less rigid

Copenhagen

DENMARK'S Minister of Environment is poised to allow the country's first deliberate releases of genetically modified organisms. Following a parliamentary debate on proposed field trials of sugar beet containing either a herbicide-resistant or a virus-resistant gene, Ms Lone Dybkjaer has indicated that she will grant the approvals that are necessary under the Environment and Gene Technology Act, Europe's only specific legislation for gene experiments. That decision is the latest sign that Denmark's three-year-old law is not necessarily as restrictive in practice as it seems on paper.

Among those most relieved by these signs are three large Danish companies with an interest in exploiting gene technology — Danisco, Novo-Nordisk and Carlsberg. Their criticisms of the law are only somewhat tempered by the increasing speed with which National Food Agency civil servants are dealing with applications, and by some amendments to the law that will come into force on 1 July.

For Novo-Nordisk, the pharmaceutical and enzyme company formed by a recent merger, the most important amendment to the 1986 law is the one that will allow pilot plants to be treated as research rather than production facilities and so be subject to less stringent regulations. A second relaxation means that a company

says that approval will be sought next year for follow-up field tests in Denmark, as well as for related trials in France and Britain. Meanwhile company scientists are working hard on other genetic modifications of both sugar beet and rapeseed.

The two genetically modified sugar beets to be field tested in Denmark next year will be the first using a commercially relevant line, Kjaegaard claims. One modification makes beet resistant to glyphosate, the active ingredient of Monsanto's herbicide Roundup, which is considered to be more environment-friendly than most alternatives but is toxic to ordinary sugar beet. The other modification is designed to confer resistance to rhizomania, a disease caused by the beet necrotic yellow vein virus.

Although scientists at the Carlsberg Laboratory have hopes of genetically modifying the barley and yeast used in the

breweries whose profits fund their research through the Carlsberg Foundation, they are not about to follow in Danisco's footsteps. One reason, says Diter von Wettstein, head of physiology, is the feeling that the public is not yet prepared to buy beer brewed with the aid of gene technology. Another is that the techniques for inserting new genes into barley are not yet perfected.

By the time they have been, Denmark's gene law may have been further relaxed. It is due for additional amendments in 1990/91. One possibility is that it will at that stage fall into line with European Community regulations. Current Danish law is still considerably more restrictive on the contained use of genetically modified organisms than what seems likely to become the minimum European law (see below).

As for deliberate release, Europe is still in disarray, with current Danish law and German intentions appearing to be the most restrictive. **Peter Newmark**

ENGINEERED ORGANISMS

Euro guidelines take shape

Munich

EUROPEAN Community (EC) environment ministers on 8 June agreed on guidelines for the use of genetically modified organisms in laboratories or production facilities, but disagreed on how to regulate their release into the environment.

The guidelines were proposed last year by the European Commission, the executive body of the EC, and later amended by the European Parliament (see *Nature* 339, 413; 8 June 1989). The meeting of the Council of Environment Ministers was a first response to the amendments suggested by the Parliament, which will hold a second reading of the proposal this autumn. The council has the final say on the content of the proposal.

The council, meeting in Luxembourg, adopted some of the stricter guidelines proposed by the European Parliament on the use of genetically modified organisms in laboratories and production facilities. Under the guidelines adopted, "dangerous" modified organisms must be licensed by national authorities the first time they are used in a laboratory and every time they are used for production. In addition, the council recommended flexible deadlines for licensing procedures in order to allow for public participation.

The final proposal reflects West German wishes for stricter regulation, which France and Britain have traditionally opposed. The ministers disagreed about whether to require community-wide licensing of products containing modified organisms and intended for release. The minister from Spain, which holds the EC presidency until next month, decided after

a brief discussion to return the proposal to a ministerial committee for further debate.

Regulations approved by the council comprise a minimum standard for EC member states, which are bound to adopt them. Member states are free to add to community regulations. **Steven Dickman**

EUROPEAN COMMISSION

A new Framework is established

Paris

TEN joint research programmes were approved by European Community research ministers last week at a meeting of the Council of the European Commission in Luxembourg, and three others were passed for a second reading in the European Parliament. Ninety-one per cent (Ecu 482 million, about US\$530 million) of the EC Framework programme for research and technological development has now been committed, and a further 5 per cent earmarked for the three programmes still to receive final ratification.

The programmes include specific efforts on coastline protection, expert systems and automated language translation, as well as general projects to assess the purpose and effectiveness of research policy and technological development in the EC.

Research ministers also heard preliminary details of a proposal to streamline the EC's five-year plan which would make it a more flexible, rolling programme. Full details are expected in mid-July.

Peter Coles



no longer has automatically to stop work when a complaint is lodged with the Environmental Appeal Board.

What has not changed, Novo-Nordisk complains, is the amount of time and paperwork involved in filing an application. Nor has there been any move towards substituting a procedure of notification for one of authorization for those organisms that are widely held to offer very little risk. Even so, as the world's largest supplier of industrial enzymes, Novo-Nordisk plans to be using gene technology to make all twenty or so enzymes that it sells within five years.

Danisco, the food conglomerate that is to field test the genetically modified sugar beet, is also keen to extend the application of gene technology. The company's director of biochemistry, Leif Kjaegaard,

Taxonomists and stability

SIR—The erroneous assumption has been made in recent critiques of the frequent name-changing by certain taxonomists that only two camps are involved: the “taxonomists, who want to change the names of organisms, and other biologists who do not” (J. A. Barnett, *Nature* **338**, 547; 1989). Others in earlier communications have made similar statements. Actually, there is a large minority (perhaps actually a majority) of taxonomists who are as anxious to retain the stability of names as the average biologist. These biologically orientated taxonomists fully realize that names are the keys to a vast information storage and retrieval system and that every change of a name corresponds to a change of a key, resulting in confusion and loss of information.

In discussing stability, one must clearly distinguish between inevitable changes in a name, such as when an incorrectly classified species is transferred to the genus to which it belongs, and changes caused only by a rigid application of certain arbitrary rules of nomenclature. To minimize the number of the latter changes, zoologists had for years a set of rules automatically protecting universally used names.

These rules were unfortunately rejected by the majority of delegates attending a nomenclature meeting at the International Congress of Zoology in 1963. Many taxonomists have since pleaded for the restoration of these stabilizing provisions. Restoring them would go a long way towards stabilizing zoological nomenclature, a goal surely supported by the majority not only of biologists but even of taxonomists. It would also greatly reduce the workload of the International Commission on Zoological Nomenclature and its secretariat.

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The grants game

SIR—A form of lying has become an accepted commonplace in science. Its systematic use may help to explain the recent rash of reports of scientific fraud.

The ‘lies’ are grant proposals. Kendrew has pointed out that in order to obtain a research grant, a scientist “has to cheat. If he expressed his own motivation honestly he would get no money”¹. Angier quotes Weinberg as another who believes that to be the case².

Kendrew and Weinberg are right. All innovative scientists know that they would rarely be funded, such is the nature of the review system, if they truthfully set out what they actually intended to do and

what their motivation was for doing it. So we all tell acceptable lies.

What do we do when faced with the necessity of telling a lie in order to achieve what we believe to be legitimate ends? All of us believe that we know the difference between *real* lying, which is unforgivable and subverts science, and *acceptable* lying which we must do so that we can obtain the funds to discover the truth. We believe that we can repeatedly lie in our grant proposals and yet know when to stop when we write up the results.

But do we? Do even the best among us, once we step over the line which says that truth and honesty are indivisible, know any more what is truly right and truly wrong? And what about those who are less than the best, who are less clear-sighted, whose self-confident morality is less robust? What does the blurring of values, the elision of acceptable and unacceptable practices, the confusion of different classes of truth, do to them? Might it not be rather difficult for them to distinguish between the acceptable lies we must tell in a grant proposal and the unacceptable ones we might tell when writing our results or when reviewing a competitor’s grant application or paper?

If we cannot do better than collude in a system which requires us to tell lies in order to obtain money to seek the truth, we deserve neither the public’s trust nor its funding.

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1. Kendrew, J. in *Priorities in Research* (eds Kendrew, J. & Shelley, J.) p. 171 (Excerpta Medica, Amsterdam, 1983).
2. Angier, N. in *Natural Obsessions: The Search for the Oncogene*, p. 24 (Houghton Mifflin, Boston, 1988).

On principles

SIR—John Barrow (*Nature* **339**, 170; 1989) calls into question our report (*Nature* **337**, 411–412; 1989) on the recent conference in Venice on the anthropic principle in cosmology. We find it difficult to see that Barrow’s letter accurately conveys the main thrust of his contribution to the conference.

Anyone seriously interested in this topic will know that there is a range of principles proposed, from the weak anthropic principle (WAP), through the strong anthropic principle (SAP), to the final anthropic principle (FAP) put forward by Barrow and Tipler in their major book¹. The first is unexceptionable and the second intriguing but highly debatable, while the last is so difficult to accept that its advocacy tends to bring the whole topic into disrepute (see for example Martin Gardner’s comments in the *New York Review*²).

At the Venice conference, Barrow gave

a wide-ranging and thought provoking talk on “Patterns of explanation in cosmology”, in which the weak anthropic principle was put forward as having a powerful explanatory role. He also commented on the strong anthropic principle, but did not even mention the final anthropic principle. Just as in Sherlock Holmes’ comment on the strange incident of the Dog in the Night (what did he do? nothing!), we believe the listener to that talk was entitled to conclude Barrow did not at that time regard the FAP as a significant principle of explanatory power in cosmology (else surely it would have been mentioned). Thus our comment that he had abandoned the FAP.

His letter to *Nature* states that he defended the FAP in later discussions arising from other people’s talks. Thus, to be technically correct, we should have merely said that he did not mention the issue in his major review. We believe the implication remains (and is of interest to your readers): a serious review of explanation in cosmology will not include the FAP. This was certainly the impression conveyed to us, and others, by Barrow’s presentation.

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1. Barrow, J.D. & Tipler, F.J. *The Anthropic Cosmological Principle* (Oxford University Press, 1986).
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Anonymous review

SIR—Alun Anderson writes: “Eliminating bias by hiding the names and affiliations of authors and the identity of reviewers had little effect on editors’ recommendations” (*Nature* **339**, 164; 1989). Most editors have such a wide range of material to deal with that they are not normally the best people to recognize bias. Authors are.

The identity or affiliation of authors has never been hidden from this reviewer, whereas reviewers’ identities are nearly always hidden from authors — at least in my experience and that of people with whom I have discussed the matter.

Bias would be more explicitly revealed if reviewers signed their names, even though it could not be eliminated. Reviewers would also write more carefully considered reports. I believe that would go some way towards eliminating errors. Then, even if papers did “make their way down the hierarchy of journals”, they would at least be corrected, modified and improved *en route*.

J.B. WRIGHT

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Valuation of an Amazonian rainforest

Charles M. Peters, Alwyn H. Gentry and Robert O. Mendelsohn

Exploitation of non-wood resources would provide profits while conserving Amazon forests. Yet little is done to promote their development.

TROPICAL forest resources have traditionally been divided into two main groups: timber resources, which include sawlogs and pulpwood; and non-wood or 'minor' forest products, which include edible fruits, oils, latex, fibre and medicines. Most financial appraisals of tropical forests have focused exclusively on timber resources and have ignored the market benefits of non-wood products. The results from these appraisals have usually demonstrated that the net revenue obtainable from a particular tract of forest is relatively small, and that alternative uses of the land are more desirable from a purely financial standpoint. Thus there has been a strong market incentive for destructive logging and widespread forest clearing.

We contend that a detailed accounting of non-wood resources is required before concluding *a priori* that tropical deforestation makes financial sense. To illustrate our point, we present data concerning inventory, production and current market value for all the commercial tree species occurring in one hectare of species-rich Amazonian forest. These data indicate that tropical forests are worth considerably more than has been previously assumed, and that the actual market benefits of timber are very small relative to those of non-wood resources. Moreover, the total net revenues generated by the sustainable exploitation of 'minor' forest products are two to three times higher than those resulting from forest conversion.

Our findings are based on an appraisal of an area along the Rio Nanay near

to the small village of Mishana (3°47'S, 73°30'W), 30 km south-west of the city of Iquitos, Peru. Annual precipitation in the region averages 3,700 mm; soils are predominantly infertile white sands. The inhabitants of Mishana are detribalized indigenous people called *ribereños* who make their living practising shifting cultivation, fishing and collecting a wide variety of forest products to sell in the Iquitos market.

A systematic botanical inventory of 1.0 ha of forest at Mishana showed 50 families, 275 species and 842 trees ≥ 10.0 cm in diameter¹. Of the total number of trees on the site, 72 species (26.2%) and 350 individuals (41.6%) yield products with an actual market value in Iquitos. Edible fruits are produced by seven dicotyledonous and four palm species, sixty species produce commercial timber and one species, *Hevea guianensis* Aubl., produces rubber. The forest also contains medicinal plants, lianas and several understorey palms of commercial importance in the region, yet these species were too small to be included in our sample.

Annual production rates for all the fruit trees and palms in our sample area were either measured by counting and weighing all the fruits produced by a sub-sample of adult trees (4 species), or estimated from interviews with local collectors (7 species). Latex yields for wild *Hevea* trees were taken from the literature². The merchantable volume of each timber tree was calculated using published regression equations relating diameter at breast height (137 cm) to commercial height³.

We collected average retail prices for different forest fruits in 1987 by making monthly surveys of the Iquitos produce market. We obtained rubber prices, which are officially controlled by the Peruvian government, from the agrarian bank office. Four independent sawmill operators in Iquitos were interviewed to determine the mill price of each timber species. All market data were converted from Peruvian intis to 1987 US dollars using an exchange rate of 20 intis to the dollar.

The labour investment associated with fruit collection and latex tapping was estimated in man days per year based on interviews and direct observation of local collecting techniques. We then calculated cumulative harvest costs using a wage rate of US\$2.50 per man day, the minimum wage in Peru during 1987. Based on previous studies conducted in Mishana⁴, we assumed that transport costs for fruit and latex are 30% of the total market value of these products. Studies by the Food and Agriculture Organization⁵ suggest that logging and transport costs in the Peruvian lowlands equal 30–50% of the total value of the timber harvested; we used a 40% extraction expense in our study.

Based on our estimates of the density, productivity and market price of each fruit tree and palm, one hectare of forest at Mishana produces fruit worth almost \$650 each year (Table 1). Annual rubber yields amount to about \$50. After deducting the costs associated with collecting and transporting this material to market, net annual revenues from fruit and latex are \$400 and \$22, respectively.

Given that both fruit and latex can be collected every year, the total financial value of these resources is considerably greater than the current market value of only one year's harvest. Clearly, the net revenue generated by all future harvests must also be estimated and included in the analysis. We used a simple discounting model to calculate the net present value (NPV) as V/r , where V is the net revenue produced each year and r is a 5% inflation-free discount rate, of these annuities. Assuming that 25% of the fruit crop is left in the forest for regeneration, the NPV of sustainable fruit and latex harvests is estimated at \$6,330 per hectare.

The Mishana forest also contains 93.8 m³ ha⁻¹ of merchantable timber (Table 2). If liquidated in one felling, this sawtimber would generate a net revenue of \$1,000 on delivery to the sawmill. A logging

TABLE 1 Annual yield and market value of fruit and latex produced in one hectare of forest at Mishana, Rio Nanay, Peru

Common name	Species	No. trees	Annual production per tree	Unit price (US\$)	Total value (US\$)
Aguaje	<i>Mauritia flexuosa</i> L.	8	88.8 kg	10.00/40 kg	177.60
Aguajillo	<i>Mauritiella peruviana</i> (Becc.) Burret	25	30.0 kg	4.00/40 kg	75.00
Charichuelo	<i>Rheedia</i> spp.	2	100 fruits	0.15/20 fruits	1.50
Leche huayo	<i>Couma macrocarpa</i> Barb. Rodr.	2	1,060 fruits	0.10/3 fruits	70.67
Masaranduba	<i>Manilkara quianensis</i> Aubl.	1	500 fruits	0.15/20 fruits	3.75
Naranjo podrido	<i>Parahancornia peruviana</i> Monach.	3	150 fruits	0.25/fruit	112.50
Sacha cacao	<i>Theobroma subincanum</i> Mart.	3	50 fruits	0.15/fruit	22.50
Shimbillo	<i>Inga</i> spp.	9	200 fruits	1.50/100 fruits	27.00
Shiringa	<i>Hevea quianensis</i> Aubl.	24	2.0 kg	1.20/kg	57.60
Sinamillo	<i>Oenocarpus mapora</i> Karst.	1	3,000 fruits	0.15/20 fruits	22.50
Tamamuri	<i>Brosimum rubescens</i> Taub.	3	500 fruits	0.15/20 fruits	11.25
Ungurahui	<i>Jessenia bataua</i> (Mart.) Burret	36	36.8 kg	3.50/40 kg	115.92
Totals		117			697.79

Fruit yields measured for *M. flexuosa*, *J. bataua*, *P. peruviana* and *C. macrocarpa*; estimated yields for other fruit trees based on interviews with local collectors.

operation of this intensity, however, would damage much of the residual stand and greatly reduce, if not eliminate, future revenues from fruit and latex trees. The net financial gains from timber extraction would be reduced to zero if as few as 18 fruit trees were damaged by logging.

Periodic selective cutting presumably would be more compatible with annual fruit and latex collection. Yield functions derived for the 60 commercial timber species at Mishana using stand-table projection and a mean diameter increment of 0.3 cm yr⁻¹ indicate a maximum sustainable harvest of about 30 m³ ha⁻¹ every 20 years. Multiplying this volume by a weighted average market price of \$17.21 m⁻³ and deducting harvest and transport costs gives a net revenue of about \$310 at each cutting cycle. Discounting a perpetual series of these periodic revenues back to the present yields a net present value (NPV = $V/(1-e^{-t})$, where t is the number of years between harvests), of \$490 for timber.

Based on the assumption of sustainable timber harvests and annual fruit and latex collection for perpetuity, we estimate that the tree resources growing in one hectare of forest at Mishana possess a combined financial worth of \$6,820. Fruits and latex represent more than 90% of the total market value of the forest, and the relative importance of non-wood products would increase even further if it were possible to include the revenues generated by the sale of medicinal plants, lianas and small palms. In view of the disproportionately low NPV of wood resources and the impact of logging on fruit and latex trees, timber management is a marginal financial option in this forest.

We acknowledge that these projections are subject to temporal changes in market prices, production levels and harvest intensities that could either increase or decrease the actual market benefits obtainable from the forest. Moreover, we realize that not every hectare of tropical forest will have the same market value as our plot in Mishana. Further studies are needed to determine how floristic composition, productivity and distance to markets influence the financial worth of a forest. Yet we believe that the NPVs calculated in this study provide a useful economic benchmark for comparing alternative land-use practices and management options for Amazonian forests.

Our results indicate that the financial benefits generated by sustainable forest use tend to exceed those that result from forest conversion. Using identical investment criteria, that is discounting a perpetual series of net revenues at a 5% interest rate, the NPV of the timber and pulpwood obtained from a 1.0-ha plantation of *Gmelina arborea* in Brazilian Amazonia is estimated at \$3,184 (ref. 6), or less than half that of the forest. Simi-

TABLE 2 Merchantable volume and stumpage value of the commercial timber tree in one hectare of forest at Mishana, Rio Nanay, Peru

Commercial name	Genera included	No. trees	Wood volume (m ³)	Mill price (per m ³) (US\$)	Stumpage value (US\$)
Aguano masha	<i>Trichilia</i>	4	0.55	14.80	4.88
Almendo	<i>Caryocar</i>	1	0.08	14.80	0.71
Azucar huayo	<i>Hymenaea</i>	1	0.10	14.80	0.89
Cumala	<i>Iryanthera, Virola</i>	83	19.77	19.00	225.38
Espintana	<i>Guatteria, Xylopia</i>	7	1.47	21.00	18.52
Favorito	<i>Osteophloeum</i>	2	3.90	14.80	34.63
Ishpingo	<i>Endlicheria</i>	4	0.82	14.80	7.28
Itauba	<i>Mezilaurus</i>	3	0.29	14.80	2.57
Lagarto caspi	<i>Calophyllum</i>	2	0.25	40.30	6.04
Loro micuna	<i>Macoubea</i>	1	1.37	14.80	12.17
Machimango	<i>Eschweilera</i>	5	0.76	20.15	9.19
Machinga	<i>Brosimum</i>	10	24.61	14.80	218.53
Moena	<i>Aniba, Ocotea</i>	6	0.75	42.00	18.90
Palisangre	<i>Dialium</i>	1	0.27	14.80	2.39
Papelillo	<i>Cariniana</i>	1	1.19	14.80	10.57
Pashaco	<i>Parkia</i>	19	4.19	14.80	37.21
Pumaquiro	<i>Aspidosperma</i>	12	10.22	14.80	90.75
Quinilla	<i>Chrysophyllum, Pouteria, Manilkara</i>	34	9.18	31.80	175.15
Remo caspi	<i>Swartzia, Aspidosperma</i>	28	11.65	14.80	103.45
Requia	<i>Guarea</i>	4	1.06	14.80	9.41
Tortuga caspi	<i>Duquetia</i>	1	0.13	14.80	1.15
Yacushapana	<i>Terminalia</i>	2	0.71	14.80	6.31
Yutubanco	<i>Heisteria</i>	2	0.53	14.80	4.70
Totals		233	93.85		1,000.78

Twenty-three commercial names represent 28 genera and 60 tree species.

larly, gross revenues from fully-stocked cattle pastures in Brazil are reported⁷ to be \$148 ha⁻¹ yr⁻¹. The present value of a perpetual series of such pastures discounted back to the present is only \$2,960, and deducting the costs of weeding, fencing and animal care would lower this figure significantly. Both these estimates are based on the optimistic assumption that plantation forestry and grazing lands are sustainable land-use practices in the tropics.

The results from our study clearly demonstrate the importance of non-wood forest products. These resources not only yield higher net revenues per hectare than timber, but they can also be harvested with considerably less damage to the forest. Without question, the sustainable exploitation of non-wood forest resources represents the most immediate and profitable method for integrating the use and conservation of Amazonian forests. Why has so little been done to promote the marketing, processing and development of these valuable resources?

We believe that the problem lies not in the actual value of these resources, but in the failure of public policy to recognize it. Tropical timber is sold in international markets and generates substantial amounts of foreign exchange; it is a highly visible export commodity controlled by the government and supported by large federal expenditures. Non-wood resources, on the other hand, are collected and sold in local markets by an incalculable number of subsistence farmers, forest collectors, middlemen and shop-owners. These decentralized trade networks are extremely hard to monitor and easy to

ignore in national accounting schemes.

The non-market benefits of tropical forests have been discussed recently^{8,9}. Tropical forests perform vital ecological services, they are the repository for an incredible diversity of germplasm, and their scientific value is immeasurable. The results from this study indicate that tropical forests can also generate substantial market benefits if the appropriate resources are exploited and properly managed. We suggest that comparative economics may provide the most convincing justification for conservation and use of these important ecosystems. □

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Can journals influence science?

Among other things, a US House of Representatives subcommittee last week raised the question whether science journals could (and should) do more to enhance the integrity of science.

Do the science journals have an influence on the way that science is conducted? And if not, should they? These apparently innocuous questions are in reality contentious, for there is a powerful school of thought, chiefly represented by the editors of journals, which holds that the scientific literature is and should be a passive means of communication — a mirror held up to the face of research in which people other than its authors can discover what is happening in laboratories the world over.

That, of course, is an idealization which is far from the truth. Even as things are, and in the general climate of the opinion that the journals should be passive instruments, journals have an important influence. Many say that they have too much. It is an important question to know whether they should have more.

The feedback link between journals and the conduct of research is readily identified. Research is incomplete if it remains unpublished. People who have slaved away for months or years on a project that never finds its way into print might just as well have dug their gardens.

That lesson is generally understood, and helps account for the mounting demand for space in the world's scientific literature. There are also more immediate pressures: people's research programmes, even their careers, may crucially depend on what their sponsors or employers see in print. It is not surprising that, within reason, authors will go to endless trouble to meet conditions laid down by journals and their editors. In the process, they are moulding accounts of their research in response to external demands.

There is every reason to believe that the influence of the publication policies of journals has already fed back so far as to influence the way in which experiments are planned. Now that most journals maintain refereeing systems and are constantly seeking ways of making them more rigorous, prudent authors naturally anticipate the issues that will be raised.

That influence alone would explain why it is rare, these days, to come across a manuscript intended for publication from a laboratory in the United States or Western Europe in which an account of experiments carried out is not accompanied by control experiments that appear, at least at first sight, to be adequate. (The same influence no doubt explains why authors from elsewhere are

now so much more often disappointed.) A glance at journals in the biomedical field spanning the past quarter of a century would make it plain that there has been, as a consequence, a perceptible change in the character of what is published: the information density is greater, articles are at once more professional and harder to understand.

Journals editors and their referees willingly take the credit for having helped bring about changes such as these, so does it not follow that they should similarly take credit for the general improvement there has been in authors' willingness to acknowledge the importance of other people's work? Of course. Referees are habitually diligent in pointing when authors have failed to acknowledge a legitimate source. Journals do indeed take the credit. So should they not, by the same test, also share some of the responsibility for researchers' failure always to follow the highest standards of professional conduct?

There, of course, lies the rub. At some level, all journals are competing for the outcome of the same pool of research, while even the most academic journals cannot afford to be seen empty some month or quarter. So they and their contributors have a mutual interest that the quality of what they publish should be improved, which is why they work together so willingly on the successive revision of the manuscripts destined to be published. But, in competitive circumstances, there are obvious risks in enquiring too closely into the ethical environment in which a piece of research has been carried out: the authors might take offence and go elsewhere.

The peer-review process has many faults (its anonymity is increasingly difficult to defend), but it is also perpetually moving to see how many senior people are willing to spend time spotting errors in other people's students' work. These days, referees can be relied upon almost without question to draw attention to control experiments that should have been carried out and references that may have been overlooked, while it is constantly humbling that they are able to quote from their experience the evidence for believing that an author's account of his experiments must be incomplete. Referees are a group of people whose unselfishness is insufficiently acknowledged.

Even so, referees are not good at spotting certain kinds of defects in manuscripts submitted for publication. They draw attention to missing references within their ken but they do not often raise questions about the propriety of an author's purported authorship, for example. Nor do referees often question the reasons why an author has carried out the piece of research submitted for publication, raising the possibility that he or she may have a hidden, even a conflicting, motive.

There is of course no reason why referees, unpaid and overworked as they are, should shoulder this responsibility, but who, if they cannot, will? There is a case that the responsibility lies with journals, and that they should shoulder it. In general, most journals are ill-equipped for such a task. These days, there is a host of similar ethical questions crying for attention — the use of animals in research, for example, or the degree to which freedom of research in genetic engineering should be attenuated by the hypothetical fear of untoward consequences. To what extent can most journals cope?

That, of course, is why it so often seems preferable to follow the principle that journals are passive entities, whose duty is discharged once a piece of research has been published. "In due course", the argument runs, "the scientific community will decide what to take seriously". Even now, there are many journals that do not entertain the idea that they should publish letters from correspondents complaining that material already published should not have seen the light of day. Others publish comments on material already published, but only after the original authors have responded. (The result is usually that outsiders cannot tell which is correct.) How can such policies be justified except by the equally self-demeaning propositions that published research has an importance of its own and that its publication is of no importance?

Journals have other reforms to effect apart from that towards openness. These days, there are few fields of science without distinctive ethical problems, but most of them are served only by specialist journals that publish only the results of researchers' work. Can that be right, or even prudent? Free governments boast that their press is free, but science that reckons to thrive on free thought is hamstrung by the pusillanimity of the bulk of its journals.

John Maddox

The sheep in wolf's clothing

Dagmar Ringe

SITE-directed mutagenesis, the *idée fixe* of modern biologists, represents a novel, powerful strategy to inspect mechanisms, to alter biochemical behaviour, and to improve the physical characteristics of proteins. The biochemist dreams of creating the perfect enzyme; if we only jiggle this or that residue a bit . . . The immunologist is thinking about chimaeric antibodies or even catalytic antibodies. The industrial biologist is counting profits from thermostable, pH-stable, FDA-resistant mutants. The pharmaceutical chemist is going to create the magic bullet from nature's own arsenal by tinkering a little with the existing shell. Some significant strides have been made in altering the properties of proteins in precisely these directions. One such example of successful enzymatic tinkering is reported on page 721 of this issue¹ by Madison *et al.*

Most experiments attempting to redesign an enzyme have produced a less specific or less active catalyst than the wild type from which it was derived. The theoreticians have had a field day with these failures, pointing to obvious flaws in the strategy of the biochemist relative to the strategy of evolution. The experiment reported by Madison *et al.* is an example of such a strategy, but with a significant difference: the authors wish to produce a less interactive enzyme. And they succeed! By changing a single residue in the enzyme tissue plasminogen activator (t-PA), the effectiveness of the interaction between it and the natural t-PA inhibitor plasminogen activator inhibitor-1 (PAI-1) is compromised. By removing a loop of

seven residues in the enzyme the interaction between enzyme and inhibitor is practically abolished, but catalytic efficiency is retained.

For those who do not read the *Wall Street Journal* with their morning coffee, t-PA is the enzyme chiefly responsible for some of the misfortunes and fortunes of several prominent biotechnology companies. The large potential market for t-PA as a pharmaceutical derives from its involvement in thrombolysis. Mammalian plasma contains an enzymatic system capable of dissolving the fibrin in blood clots. One component of this system, t-PA, generates the active enzyme plasmin by limited proteolysis of plasminogen. Plasmin degrades the fibrin network of a clot to form soluble products. It is therefore a potential thrombolytic agent for the treatment of pulmonary embolism, deep-vein thrombosis, heart attacks and strokes. t-PA is a complex, multidomain enzyme made up of repeating units called kringles, which bind to fibrin, and a proteolytic domain. The efficiency of t-PA *in vivo* is limited by the presence of PAI-1, which binds to the protease domain, blocking the active site. The objective of the study of Madison *et al.* was to engineer an improved version of t-PA which would be less sensitive to PAI-1.

Madison *et al.* chose sites for mutations on the basis of a modelling 'experiment' which predicts that a certain loop, and especially one residue within it, forms part of the interactive surface between the enzyme and the inhibitor. What is noteworthy is that no three-dimensional

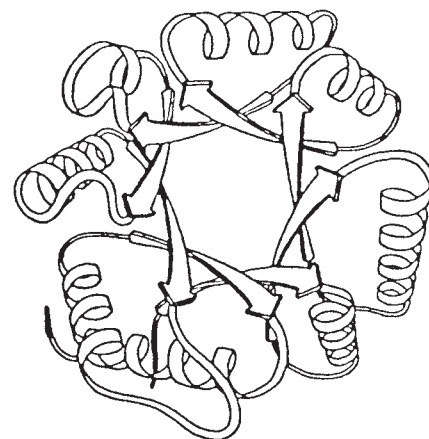


FIG. 1. Schematic drawing of triose phosphate isomerase showing the eight-stranded α/β -barrel (drawing by J. Richardson).

structure is known either for t-PA or for PAI-1. The residues changed were identified on the basis of sequence similarity between t-PA and the digestive enzyme trypsin, whose structure is known. Moreover, the structure of the complex between trypsin and a naturally occurring inhibitor, bovine pancreatic trypsin inhibitor, is also known, allowing identification by analogy of the most likely residues at the interaction surface between t-PA and PAI-1. All of which is very convenient if one wishes to make a more effective t-PA. However, the fact that the structure of t-PA resembles the structure of trypsin, and that this resemblance can be used to make predictions about interactions between the enzyme and its inhibitor, illustrates a point that has recently been shown to have much broader implications for protein engineering.

The technique of site-directed mutagenesis has been highly successful in assessing the roles of individual amino acids in enzymatic mechanism², stability of proteins to thermal inactivation³, and stabilizing tertiary structure during the folding process⁴, to mention a few. Whole segments have been moved successfully from one protein to another, for practical purposes such as antibody development⁵ and for studies of structural stability⁶.

The sum experience of these experiments is that protein structures are remarkably stable to tinkering but their functional properties are alarmingly fragile. Catalytic specificity and efficiency are the result of the precise configuration of many different amino acids, and as such are extremely sensitive to small changes in geometry. Such changes can be produced by substitutions quite far removed from the active site itself, even if they leave the general architectural features of the protein intact. In fact, most mutations *do* leave the architecture intact. One is struck by the fact that radical changes of amino-acid residues and concomitant changes in the physical properties of the side chains (charge, size and so on) can easily be

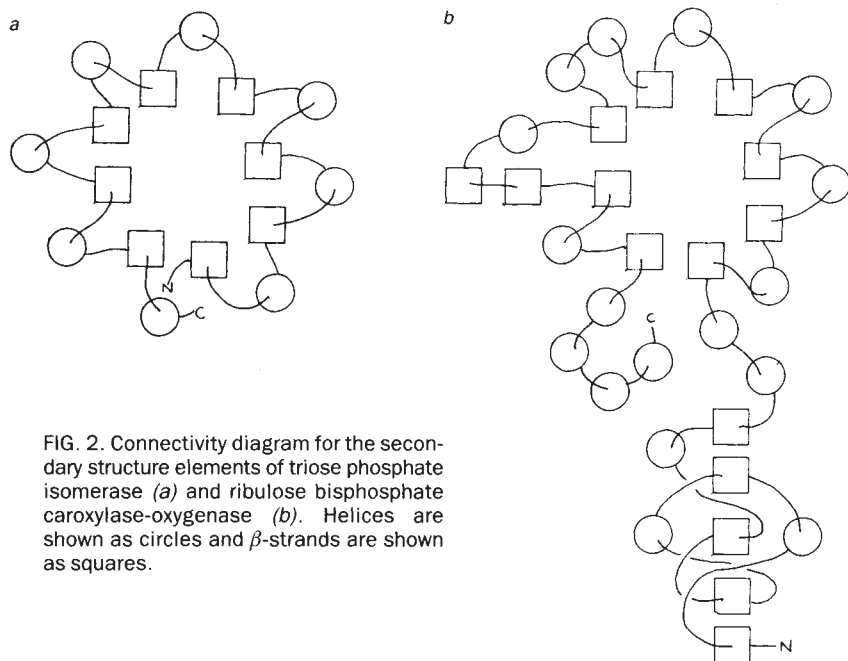


FIG. 2. Connectivity diagram for the secondary structure elements of triose phosphate isomerase (a) and ribulose biphosphate carboxylase-oxygenase (b). Helices are shown as circles and β -strands are shown as squares.

accommodated within a protein structure. To be sure, it is a common experience of protein engineers that some mutations so destabilize the protein that it is rapidly degraded in the cell before it can be purified. But even in such cases, it is possible that the folded form, though too transient to be observable, does not differ drastically from the native conformation. The overwhelming majority of mutations leave the gross structural features intact.

The reason for this may be the existence of a relatively small number of highly stable folding motifs that form the basis for globular protein structures. Nature has a short menu, but the items on it seem to be quite reliable. This fact makes excellent evolutionary sense, for it allows the exchange of loops and domains from one protein to another with concomitant hybridization of functionality, since the host structure is preserved.

An excellent example of this is provided by the eight-stranded β -barrel enzymes, of which triose phosphate isomerase is the prototype (Fig. 1). Seventeen different enzymes have been found to possess this remarkably symmetrical fold as part of their three-dimensional structure. In the case of triose phosphate isomerase and some other of these enzymes, the motif occurs in its most elemental form: a strand of parallel pleated sheet followed by an α -helix, and the pattern is repeated seven more times. Joints between adjacent segments are provided by loops of varying lengths. But in many of these β -barrel enzymes large, independently folded domains are inserted into the motif at one or more of the loop positions (Fig. 2). The barrel structure is apparently able to tolerate such an insertion because of the intrinsic stability of the hydrogen bonds between the staves of the barrel (strands of sheet) and the interactions between staves and the surrounding bands of helices.

Dramatic evidence for the robustness of this motif has recently been provided by Luger *et al.*⁷. These authors took a simple $\alpha\beta$ -barrel protein, phosphoribosyl anthranilate isomerase, and circularly permuted the structure by joining the amino and carboxy termini and opening the chain at one of the loops. This engineering was done at the level of the gene. The resulting proteins not only folded into stable and unique three-dimensional structures, they also had nearly full catalytic activity, indicating that the covalent changes necessary for the rearranged strand order produce structural changes which are small or localized enough for the geometry of the active site to remain practically unaffected. This concept of stable structural architecture extends to other protein structural motifs. Which is probably the reason for the surprising success of some of the early attempts at *de novo* protein design, such as the four-helix

bundle protein of de Grado *et al.*⁸.

Dramatic demonstration of the stability of the four-helix framework is offered in recent work on the small nucleic-acid binding protein, ROP, by Tsernoglou and colleagues⁹. ROP is a small protein consisting of two antiparallel helices connected by a short turn. It dimerizes to form the functional four-helix bundle in its active form. In the experiment, those residues which form the turn were deleted, with the expectation that a single contiguous helix would result. This is not what happens: the four-helix motif is so stable that a new turn is formed from residues that used to be part of the helices, and the original structure, albeit with shorter helices, is maintained. Protein evolution may have occurred through similar, though accidental events.

All of which suggests that protein engineers, with their predilection for

conservative replacements, may be too timid. In the future it may be possible to make multifunctional enzymes by grafting widely differing structures into the middle of, as well as to the ends of, other stable proteins. t-PA itself, with its kringle and protease domains, is clearly the result of precisely such a process. All this raises the spectacle of a bizarre, man-made biochemical bestiary in which wolves with fleece pursue sheep with fangs. □

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SPACE PHYSICS

The birth of a plasmoid

Mark Saunders

THE violent disruptions, called substorms, which occur a few times each day in the centre of the Earth's huge cylindrical magnetotail, must rank among the most spectacular phenomena in magnetospheric physics. A substorm releases vast (10^{15} J) quantities of energy leading to brilliant auroras at the Earth and the catapulting of a plasmoid — a giant bubble of hot magnetized gas — into interplanetary space. The existence of plasmoids was confirmed in 1984 through satellite data and their birth is thought to involve a process termed magnetic reconnection, but the precise details are poorly understood. R. L. Richard *et al.* now present (*J. geophys. Res.* **94**, 2471–2483; 1989) magnetohydrodynamic computer simulations highlighting the relevance of the magnetic-island coalescence instability for magnetotail reconnection. In particular, their simulations suggest that plasmoid formation can occur through the coalescence of several

magnetic islands. This result may have wider significance, as plasmoid-like phenomena are observed elsewhere in the Solar System, for example in the tail of comets (comet-tail disconnection events) and in the Sun's atmosphere (coronal mass ejections).

Substorms are the means by which the Earth's magnetotail, the comet-like wake resulting from the solar wind's interaction with the Earth's magnetic field, intermittently releases large amounts of stored magnetic energy. They last about an hour and differ from geomagnetic storms which persist for a day or more and arise when the blast wave from a solar flare hits the magnetosphere. Figure 1 illustrates the sequence of magnetic field reconfigurations which are thought to occur in the plasma sheet, the central portion of the magnetotail, during a substorm. This is known as the neutral-line model of substorms. Initially (Fig. 1a) there is a distant

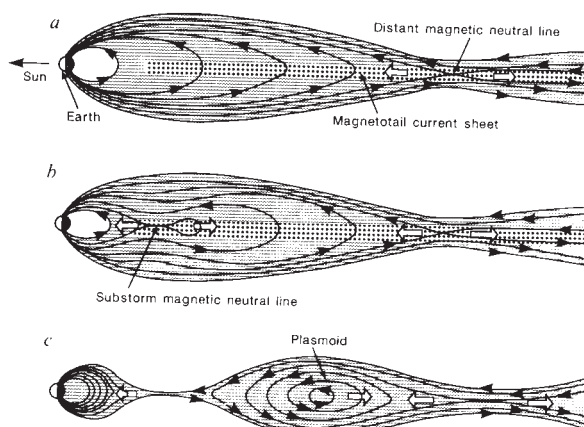


FIG. 1 Sketches of the central portion of the Earth's magnetotail in the noon-midnight meridian plane showing the chain of dramatic disruptions, culminating in the ejection of a plasmoid, which accompany a substorm. Solid lines are magnetic field lines; open arrows indicate the direction of plasma flow. (After E. W. Hones Jr *Scient. Am.*, 32–39; March 1986.)

magnetic neutral (null) line situated about 100 Earth radii ($R_E = 6,400$ km) downstream from the Earth which separates plasma sheet field lines of opposite polarity. At the start of the substorm a near-Earth or 'substorm' magnetic neutral line forms (Fig. 1b) about 15 R_E downstream from the Earth, leading to the rapid disconnection of a giant bubble of hot plasma threaded and held together by closed loops of magnetic field. This remarkable structure, a plasmoid, has a length of around 75 R_E , a width of 20 R_E and a height of around 12 R_E . It is propelled downstream by a magnetic slingshot force at speeds of around 500 km s⁻¹ (Fig. 1c).

The process responsible for the magnetic neutral lines and for the magnetic slingshot force is magnetic reconnection — a concept formulated in the 1950s by solar physicists attempting to explain solar flares. Magnetic reconnection releases magnetic energy into plasma jets and particle heating and its key features are illustrated in Fig. 2a. The process occurs when two plasma regions having oppositely directed magnetic fields are pushed together at a current sheet. Provided these plasmas have finite resistance, the field lines may break and reconnect in new combinations, resulting in a magnetic null situated where the field lines apparently cross in the magnetic field. Magnetic energy is converted into plasma motion (labelled 'jets') when the reconnected field lines are catapulted away by the magnetic tension force acting at the hairpin bend in the field lines.

Despite much research, the detailed characteristics of the reconnection process remain elusive. In a widely held view, the current sheet initially breaks up spon-

taneously into a series of magnetic islands or current filaments having a loop-like magnetic field (Fig. 2b, time t_1). This configuration is unstable to the coalescence instability (J. M. Finn & P. K. Kaw *Phys. Fluids* **20**, 72–78; 1977) by which the magnetic islands move towards each other owing to the attractive force of parallel currents (time t_2). With resistivity, this instability leads, through reconnection, to the merging or coalescence of neighbouring islands (time t_3).

Satellite observations of current filaments and plasmoids in the Earth's magnetotail current sheet led Richard *et al.* to investigate whether plasmoids could form through the coalescence of several current filaments, rather than directly through the large-scale reconnection at a neutral line implicit in the neutral line model (Fig. 1). They used a computer simulation, using the standard magnetohydrodynamic and Maxwell equations to simulate numerically the two-dimensional plasma behaviour implied by these governing equations. Quantities calculated included the plasma density, plasma pressure, flow velocity, magnetic field and current density. Two types of system were investigated: a current sheet containing an infinite chain of islands and one where the island chain was finite. The former case is relevant for studying how the coalescence process might occur in a local region of the magnetotail current sheet, and the latter is appropriate for examining the larger-scale consequences of coalescing islands and is of more immediate interest here.

The simulations were initialized by having a chain of magnetic islands whose size decreased down the magnetotail current sheet. Coalescence soon leads to the

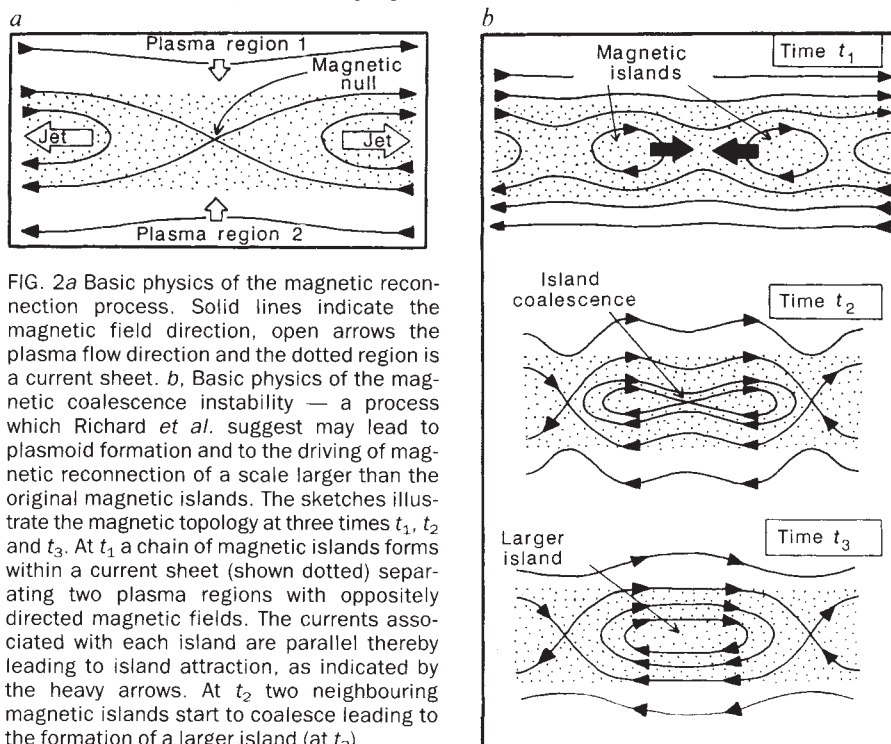


FIG. 2a Basic physics of the magnetic reconnection process. Solid lines indicate the magnetic field direction, open arrows the plasma flow direction and the dotted region is a current sheet. b, Basic physics of the magnetic coalescence instability — a process which Richard *et al.* suggest may lead to plasmoid formation and to the driving of magnetic reconnection of a scale larger than the original magnetic islands. The sketches illustrate the magnetic topology at three times t_1 , t_2 and t_3 . At t_1 a chain of magnetic islands forms within a current sheet (shown dotted) separating two plasma regions with oppositely directed magnetic fields. The currents associated with each island are parallel thereby leading to island attraction, as indicated by the heavy arrows. At t_2 two neighbouring magnetic islands start to coalesce leading to the formation of a larger island (at t_3).



100 years ago A chimpanzee's humour

IN a recent lecture Mr. Romanes is reported as having strongly denied the existence of even a trace of any feeling of the ludicrous in the renowned chimpanzee "Sally".

Being alone with a friend in Sally's house, we tried to get her to obey the commands usually given by the keeper. I said "Give me two straws, Sally." At first she appeared to take no notice. I repeated the request with no further result; but on a second or third repetition she suddenly took up a *large bundle* of straw from the floor and thrust it through the bars at us, and then sat down with her back to us. Our request was perhaps unreasonable, seeing that we had no choice morsels of banana with which to reward her. She did not, however, seem ill-tempered at our presumption, and the next instant was as lively as ever. It seems to me that her action on this occasion certainly came very near to an expression of humour. Rather sarcastic humour perhaps it was, but she certainly appeared to take pleasure in the spectacle of something incongruous, and this surely lies at the base of all sense of the ludicrous.

HAROLD PICTON.

From *Nature* **40**, 224; 4 July 1889.

formation of a large ($50 R_E \times 4 R_E$) magnetic island, or plasmoid, whose size is much greater than the sum of the areas of the original coalescing filaments. The simulations also show that the motion of this plasmoid down the magnetotail leads to magnetic reconnection being established at a magnetic neutral line on a scale much larger than the original filament size. These findings are repeatable for a wide range of initial values of the plasma resistance, viscosity and ratio of plasma to magnetic-field pressure.

Richard *et al.* focus on an interesting question: how is a plasmoid born? Not all their results are new but the paper provides the best argument for plasmoids forming through the coalescence of magnetic islands. As plasmoids are three-dimensional structures, further work is clearly necessary to extend the present simulations to three dimensions. It is also necessary to demonstrate that coalescence occurs sufficiently fast to match the 5–10 minute timescale for the expansion phase of a substorm. Further *in situ* observations of current filaments in the magnetotail current sheet are also desirable, to provide a more solid empirical base for the simulations. If such structures are indeed common, Richard *et al.* have made a useful contribution to the exciting field of substorm research. □

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CALCIUM-BINDING PROTEINS

The search for functions

John Rogers

THE concentration of calcium ions in cells fluctuates in a narrow range between resting and damaging levels, suggesting that the proteins which bind calcium are important in many biochemical regulatory processes. Calcium-binding proteins whose roles are known, such as calmodulin and troponin C, are now far outnumbered by those whose roles are not. These proteins and their possible functions were the subject of a recent meeting*.

The prototype of the calcium-binding protein superfamily is calmodulin, which not only regulates many enzymes in its calcium-bound form but also preferentially binds some proteins in its calcium-free form. One of these, neuromodulin, is identical to the neuron-specific phosphoprotein GAP-43, found mainly in neuronal growth cones and regions of synaptic plasticity (K. A. Alexander *et al.* *J. biol. Chem.* **263**, 7544; 1988). It could therefore be involved in sensitizing growth cones to regulation by calcium and protein kinase C.

In contrast to calmodulin, there are several abundant calcium-binding proteins which have not yet been shown to interact with any other protein. They tend to be regarded as calcium-buffering proteins, protecting cells against excess calcium, but this may be only one of many functions. One of these proteins is parvalbumin, most abundant in fast-twitch muscles, where it is thought to sequester cytosolic calcium after it has triggered contraction. But was this the original function of this protein? A parvalbumin-like protein called oncomodulin, previously isolated from tumours, is normally expressed in the early embryo and trophoblast, and can activate some of the same enzymes as calmodulin (J. MacManus, National Research Council, Ottawa). The oncomodulin gene has itself evolved in an unusual way in rats, by acquiring a new promoter which is an inserted retrotransposon sequence — a long terminal repeat sequence from the 'intracisternal A-particle' (IAP) family (MacManus; M. Berchtold, University of Zürich). This rat gene is expressed in greater quantities than the human equivalent, which does not have the retrotransposon promoter, but the tissue pattern of expression seems to be unchanged.

Although parvalbumin was discovered in muscles, it is also abundant in certain neurons, mainly those that are fast-firing, metabolically active and GABA-containing (C. Heizmann, University of Zürich; M. Celio, University of Kiel and others). The most surprising and controversial finding

reported at the meeting was that virtually all long ascending neurons of all sensory systems contain parvalbumin — but only in the extremities of axons (Celio). This implies more localized transport and function than previously suspected for proteins of this superfamily.

Questions of function are also raised by two other calcium-binding proteins found in some neurons, calbindin-D28 and calretinin (see figure), for which neuroanatomical surveys have failed to reveal any simple correlations with cell



Two calcium-binding proteins in neurons of chick cerebellum — calbindin-D28 (green) and calretinin (red). (Antiserum from D.E.M. Lawson.)

properties. R. J. P. Williams (University of Oxford) made a distinction between the expected properties of parvalbumin and calbindin in neurons. Parvalbumin could be a slow but high-affinity 'calcium sink', taking up the calcium that enters the cytosol during intense neural activity, whereas calbindin could bind and release calcium more quickly, acting as a 'diffusion catalyst' to redistribute it in the cell. But whether such redistribution would be necessary is an issue that was not resolved; K. Baimbridge (University of Vancouver) argued that calbindin acts mainly as a buffer. Indeed, when hippocampal pyramidal neurons maintained in cell culture are overstimulated with the transmitter glutamate, only the calbindin-negative ones show increases in calcium above 1 μ M, and most of them die, whereas most calbindin-positive neurons survive (Baimbridge).

Many participants believed that there must be more to these proteins than merely binding calcium, as suggested by their patterns of neuroanatomical distri-

bution and by the sequence comparisons of calbindin-D28 and calretinin from birds and mammals: their evolutionary rate is low and the most conserved regions are outside the calcium-binding sites (M. Parmentier, Université Libre de Bruxelles; my own work). Although the abundance of calbindin should swamp any changes in calcium concentration in the cell as a whole, the picture might be different near calcium channels. Here, calcium-binding proteins might not only limit the domain of calcium action, but might also encounter enough calcium to enable them to perform regulatory functions.

The calcium-binding protein superfamily includes a distinct group of smaller proteins that have only two calcium-binding sites. These include the two S-100 proteins of glial cells, for which a bewildering range of *in vitro* activities has been reported, from neurite-promoting activity on neurons to inhibitions of various protein phosphorylation events, including inhibition of phosphorylation of GAP-43 and of calpactin (R. Donato, University of Perugia; J. Baudier, CNRS, Strasbourg). It is not known which of these occurs *in vivo*.

In addition to the S-100 proteins, the group includes intestinal calbindin-D9 and five other proteins present in many tissues and discovered independently several times each (for example, see the sequence alignment by P. Masiakowski and E. M. Shooter in *Proc. natn. Acad. Sci. U.S.A.* **85**, 1277; 1988). First is p11, a small subunit associated with the tyrosine kinase substrate calpactin (V. Gerke, Max Planck Institute, Göttingen). Second is calcyclin, which (like the S-100 proteins) can also associate with calpactin or a related molecule, as well as being able to dimerize itself (J. Kuznicki, Nencki Institute of Experimental Biology, Warsaw). Calcyclin is induced in several cell types by growth factors. Third is p9Ka, induced in many cell types by growth factors (R. Barraclough, University of Liverpool); its messenger RNA has been independently cloned in a study which found a strong correlation of expression with metastatic potential in tumour cells (E. Lukanidin, Institute of Developmental Biology, Moscow). Fourth and fifth are a pair of calcium-binding proteins (described by several speakers) which form about half the soluble protein of granulocytes; they can join in a dimer and are variously named MRP-8 and MRP-14, cystic fibrosis antigen, L1 or calgranulins. They can be isolated as a complex with 'macrophage migration inhibitory factor' (K. Odink *et al.* *Nature* **330**, 80; 1987). A fragment of one of them is identical to a previously described 'neutrophil immobilizing factor' (J. Edgeworth, Imperial Cancer Research Fund, London), the sequence of which was published six years ago (K. Watt *et al.* *Immunology* **48**, 79;

* First European Symposium on Calcium-Binding Proteins in Normal and Transformed Cells, organized by R. Pochet, Brussels, Belgium, 20–22 April 1989.

1983). These associations might suggest a function of these proteins in inflammation; on the other hand, when granulocytes are dying and releasing 'inhibitory factors', perhaps it is not surprising that the most abundant cytosolic proteins show up among them.

Doubt also remains about the 'function' attributed to a different calcium-binding protein family, the calpactins or lipocortins. Their inhibitory action on phospholipase A_2 , and thus on the synthesis of

prostaglandins, may be due merely to sequestration of the substrate (F. Russo-Marie, INSERM U129, Paris). So in each of these families, there is an embarrassing number of abundant proteins which interact with calcium and sometimes with other second-messenger systems, and an increasing need to know what these interactions are for. □

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SPIN-GLASS THEORY

Correcting errors with glasses

H. G. E. Hentschel

N. SOURLAS points out an unexpected relationship between spin glasses and error-correcting codes on page 693 of this issue¹. Spin glasses can be used as machines to correct automatically errors occurring during transmission in a message if the message is properly encoded before being sent; under special circumstances they can perform this task perfectly. The perhaps counter-intuitive idea that a spin glass, a statistical system, can be used to erase errors rather than enhance them is due to their unusual properties at low temperatures.

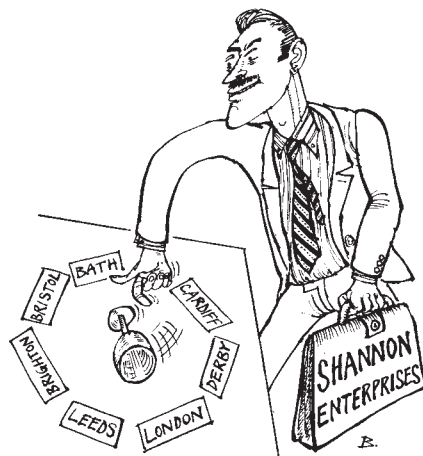
The notion of spin glasses was originally introduced by Edwards and Anderson² to account for the unusual magnetic properties of certain disordered metallic mixtures. In these materials the coupling constants between the spins on individual ions are random variables, taking positive and negative values with equal likelihood as a function of space. Their relationship to normal glass lies in the fact that both are disordered materials interacting with quenched random couplings.

Such spin-glass models exhibit unusual magnetic behaviour. At high temperatures the spins flip ceaselessly. But as the temperature is reduced each spin becomes fixed in a direction depending on the coupling constants and the positions of the other spins — a phase transition to a spin-glass phase has occurred. There are many such stable positions of the spins for a given set of coupling constants; these are the attractors of the spin dynamics, and depending on the information encoded in the coupling constants these spins can be used to perform various tasks.

For instance, it was recognized by Hopfield³ that a spin glass could describe an associative memory: encode the memories to be recalled in the coupling strengths of the network and let the spins respond to these couplings. If the initial conditions of the dynamics are close enough to one of the encoded memories, the asymptotic behaviour of the dynamics will fix the spins in positions representing the complete recalled memory — the

stored memories are the attractors of the dynamics. Of course too many memories should not be stored, otherwise recall becomes poor.

In fact, a spin glass can be transformed into a general network capable of computing the solution to a large variety of optimization tasks⁴ varying from the placement and wiring of components on a chip to the 'travelling salesman' problem, in which the task is to find the minimal tour of N cities, each city being visited



once only. Now spin glasses have found a new use — in information theory.

Information theory, initiated by Shannon⁵, relates the information content of a message to its probability of occurrence and examines questions about the transmission of coded information through noisy channels. For example, can a proper choice of code ensure that the errors in a transmitted coded message be made arbitrarily small? Shannon showed that the answer depends on the relative values of the information content of the message and the capacity of the channel. The capacity of the channel is the maximal amount of information transmissible per unit time without error, and increases with the power of the signal while decreasing with the noise.

Shannon's theory does not answer the question of what is the proper way of

coding the message to minimize transmission errors. But one possibility is to try error-correcting codes. These achieve their goal by encoding the same message in a variety of ways before transmission. This information would be degenerate if there was no noise in the channel, but with noise the additional information can be used either to check for errors, or even to correct the error if the degenerate information can identify a single error uniquely. What Sourlas shows in this issue¹ is that a class of error-correcting codes can be created where the resulting code after transmission can be identified with the coupling strengths of a spin glass. This spin glass then acts as a machine which corrects some or all of the corruption present; the probability of error still remaining after error correction can be explicitly calculated because the error-corrected message is the attractor of the spin dynamics.

Explicitly, if the message consists solely of a string of bits with values all set to 1 (as Sourlas notes, the error probability is independent of the message transmitted) then the problem of calculating the error probability can be transformed to the task of calculating the magnetization m of this spin glass. This calculation can be performed analytically, and the error probability is simply $(1 - m)$. Sourlas analyses a class of models which vary according to the additional degenerate information chosen as the error-correcting terms. One of these models, Derrida's random-energy model⁶, can then be shown to have a magnetization $m = 1$ for all signal-to-noise ratios greater than some critical value and consequently this model behaves as an ideal error-correcting code: all transmission errors can be corrected and Shannon's bound on the capacity is saturated.

These results are asymptotic and should not be regarded as solving the problems inherent in the transmission of real messages in noisy channels. For the ideal behaviour to be observed, the messages must be very long and though the coding scheme works for very low signal-to-noise ratios, the number of additional checking bits required becomes enormously large. Nevertheless, this paper does represent one further example of the growing influence of spin-glass theory⁷ in branches of science that were previously unrelated. □

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Neuropeptides find a role?

Anne W. Mudge

In the 20 years since Leeman and her colleagues purified substance P, the first known 'neuropeptide', more than 36 neuropeptides have been identified but, for the most part, their normal functions in the mature and developing central nervous system remain uncertain. On page 701 of this issue¹, Denis-Donini demonstrates a clear neurotrophic role for the neuropeptide calcitonin gene-related peptide (CGRP): it acts as a differentiation signal for dopaminergic interneurons in the olfactory bulb.

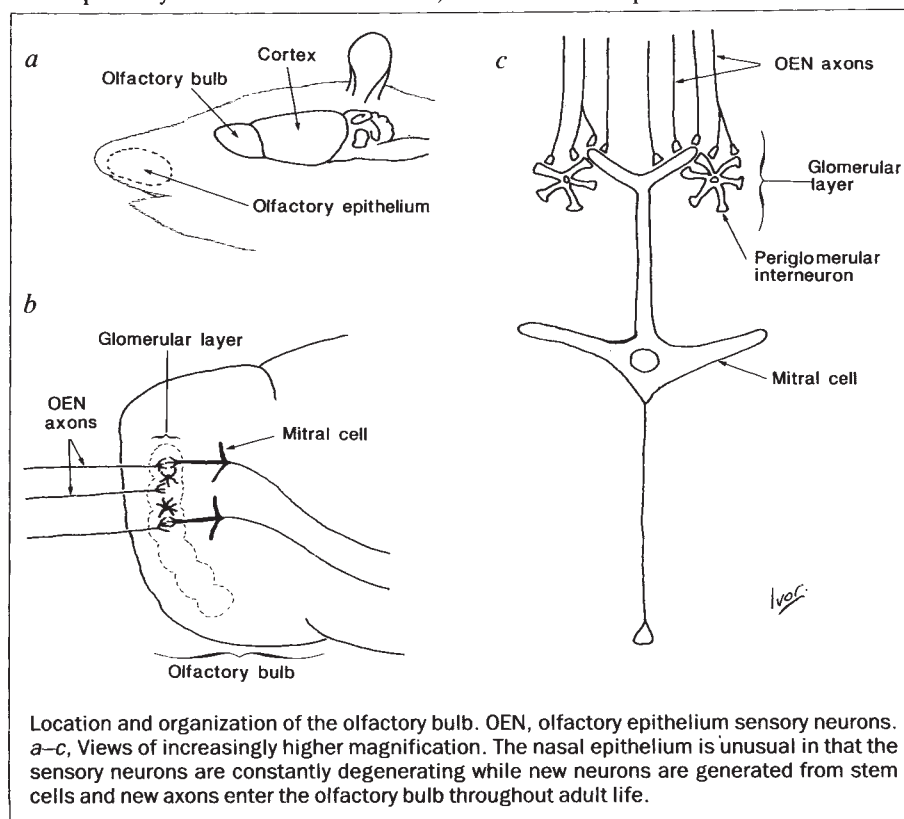
Neuropeptides are small (2–40-amino-acid residue) peptides that are stored within vesicles in nerve terminals, are released by electrical activity, and have some biological activity. They act locally as extracellular signalling molecules that can affect postsynaptic membrane potentials, secretion and/or smooth muscle contraction. Although these actions have been most clearly documented in the peripheral nervous system, they probably act similarly in the central nervous system — administration of neuropeptides into the central nervous system also often results in dramatic behavioural changes.

In general, it is thought that neuropeptides are stored in large, dense-cored secretory vesicles, which release their contents by exocytosis in response to high-frequency nerve stimulation. Once released, neuropeptides usually act more slowly and for a longer time (seconds or minutes) than classical fast neurotransmitters such as glutamate, and can diffuse away to act at a distance on neurons with appropriate receptors modulating their response to fast neurotransmitters (see refs 2,3 for reviews). In addition, it has been suspected that some neuropeptides have much longer-term, 'trophic' effects lasting for days. Although such effects have been demonstrated *in vitro* (see, for example, ref. 4), their *in vivo* significance has been hard to assess.

A major strength of the observations of Denis-Donini in this issue¹ is that, although they are made *in vitro*, they are founded on a well-documented effect *in vivo*. Sensory neurons in the olfactory epithelium are known to synapse on dopaminergic interneurons in the glomerular layer of the olfactory bulb (see figure). In rats, these periglomerular interneurons first express both tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and a dopamine-uptake mechanism at around the time of birth, after the axons of olfactory sensory neurons reach the bulb. Transection of these axons in adult rats results in the disappearance of both the enzyme and the uptake system. Denis-

Donini shows that neither olfactory bulb cells nor olfactory sensory neurons express dopaminergic properties when cultured separately, but when cultured together, both TH and the dopamine-uptake system are induced in cells with the morphology of olfactory bulb periglomerular interneurons. Moreover, addition of an extract of olfactory nerve, which contains the axons of the olfactory sensory neurons, also induces TH and the dopamine-uptake system in the bulb neurons;

of CGRP and respond normally when it is eventually added. It is unclear, however, whether TH and the dopamine-uptake system are induced *de novo* or their expression simply increased. It is also not clear whether the interneurons make an alternative transmitter in the absence of CGRP, in which case the peptide would be inducing a transmitter switch in the way that a cholinergic factor has been shown to induce adrenergic sympathetic neurons to become cholinergic⁵; the effect of CGRP, however, occurs more quickly. Because some periglomerular interneurons also make the neurotransmitter GABA, and sometimes GABA and dopamine coexist in the same



this suggests that the interaction between the sensory neurons and bulb interneurons depends on a soluble factor(s).

Denis-Donini tested the effects of two neuropeptides, substance P and CGRP, which are known to be present in axons of olfactory sensory neurons. It turns out that synthetic CGRP mimics the effects of sensory neurons or nerve extract whereas substance P does not. Finally, she showed that an antiserum against CGRP blocks the inductive effect of adding olfactory sensory neurons to bulb cell cultures, demonstrating that this peptide is required for the interaction.

In its trophic action on dopaminergic interneurons, CGRP apparently acts as a differentiation signal, rather than as a survival factor. By delaying the addition of olfactory epithelium neurons or CGRP for 4 days, Denis-Donini showed that the bulb interneurons survive in the absence

neuron⁶, it would be interesting to measure the effects of CGRP on GABA-related properties.

The findings of Denis-Donini raise the question of whether other neuropeptides are important for neuronal development in the central nervous system. The most convincing way of finding out is to study the effects of removing or inactivating the candidate neuropeptide, as so elegantly demonstrated by Denis-Donini in the olfactory bulb. □

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Goings on between the stars

Virginia Trimble

EMPTY space is both more crowded and more interesting than you might have thought; so concurred the participants at a recent meeting*. Among the many topics on which new results were presented, three that stood out were: first, fine-scale structure in the interstellar gas; second, the distribution of gas in space and among temperature-density phases; and third, rates and circumstances of star formation.

Suppose you walked the length of an American city block (1 per cent of the scale height of the Earth's atmosphere) and found that the density of air had changed by a factor two or more. Or you went up a few miles in a balloon, and the prevailing wind speed and direction changed a dozen times (while your colleague in a balloon 100 m away also saw a dozen wind components, but not always the same ones). You would be surprised. Yet the interstellar medium (ISM), with a vertical scale height of about 100 parsecs (pc) and an average density of about one atom per cubic centimetre, is highly inhomogeneous on scales of 1 pc or less.

Analogous to the balloon-ascent case, the absorption of starlight along a single line of sight through the ISM can easily require eight or more components with different gas velocities and densities to fit the observed line profiles (Laura Danly, Space Telescope Science Institute). And two sight lines separated by only a few minutes of arc, in the direction of supernova 1987A and nearby stars in the Large Magellanic Cloud, show rather different components (G. Vladilo, Osservatorio Astronomico di Trieste). Guido Münch (Max-Planck Institute (MPI) für Astronomie, Munich) noted, first, that the phenomenon is a common one, showing up for the sight lines to visual binary stars and, second, that the higher the spectroscopic resolution used, the more components revealed.

Molecular gas acts like the mythical air on the short walk. Y. Murata (Univ. Tokyo) showed the distribution of NH_3 and CS molecules in part of the Orion Nebula which are clumpy on scales of 0.05 pc and 1 km s⁻¹. John Bally (AT&T Bell Laboratories) has found similar structure, with the morphology of filaments and bubbles, in the CS distribution in other nearby clouds as well.

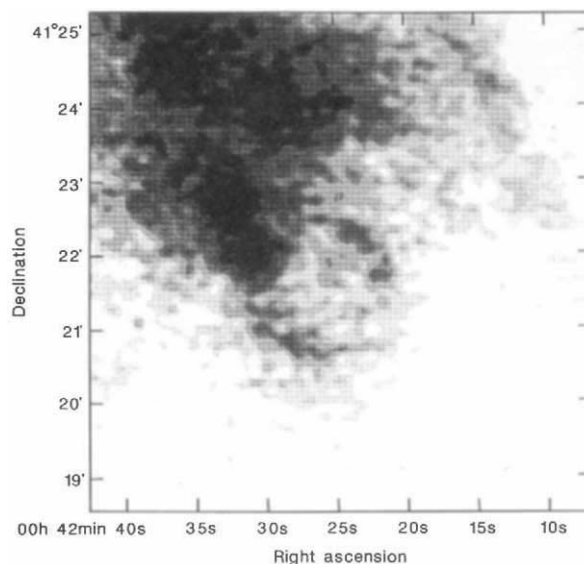
On larger scales, we can ask at least two questions about the distribution of the ISM. First, how does its average density change with position in the Galaxy? And second, how is it divided among phases of different temperature and density around

the average? Quite remarkably, a large number of speakers agreed that the basic morphology is a thin disk (scale height about 100 pc and density near the sun about 1 atom cm⁻³) in which most of the gas mass is neutral, and a lower-density thick disk (scale height 1–2 kpc) in which most of the gas is ionized (Carl Heiles, Univ. California, Berkeley; Klaas S. de Boer, Univ. Bonn; Michal Rozyczka, Warsaw Univ. Observ.; Franz Kahn, Univ. Manchester; Donald Cox, Univ. Wisconsin). The interface is wiggly, according to Cox.

For instance, de Boer noted that only 15 per cent of the neutral hydrogen (H I), but most of the highly ionized gas (Si IV, C IV, and so on) is more than 1 kpc from the

Heiles, Rozyczka, Kahn and Cox. The voting was two yeses, three noes and two maybes. The significance of additional driving of the gas by cosmic rays inside the bubbles was emphasized by Kahn, Völk and H. Bloemen (Leiden Observatory), who noted that the association of some high-velocity clouds with Cos B γ -ray sources is evidence for it. I. Felix Mirabel (Univ. Puerto Rico) suggested that about 10 per cent of the highest-velocity neutral hydrogen is falling into the Galaxy with positive total energy. Such gas presumably occupies a roughly spherical volume, but it never had the problem of getting out of the disk.

Within the disk, most of the mass is clearly in dense clouds of cold molecular and neutral gas. These occupy rather little of the total volume. The disputed issue is how to divide the remaining (small) mass and (large) volume among warmer diffuse



Radio map of an area measuring 1,200 × 1,200 parsec (pc) in the northeastern spiral arm of the Andromeda galaxy. It shows the distribution of neutral hydrogen moving at a heliocentric velocity of -115 km s^{-1} , the density increasing with the intensity of shading. The circular feature at the centre of the figure is thought to enclose a spherical cavity 330 pc in diameter (15 mm on the page) blown out by a stellar association over 20×10^6 years old. Induced star formation in the spherical shell is revealed by bright H α emission and the numerous O-, B- and Wolf-Rayet stars there. The energy required to create the shell is thought to be about 5×10^{51} erg. (Map courtesy of E. Brinks, R. Braun and S. W. Unger.)

galactic plane, and there are probably few high-velocity clouds of H I outside 1.5 kpc. From an optical and ultraviolet vantage point, Danly and C. Elise Albert (US Naval Academy) showed that a significant number of absorption line components arise between 0.3 and 1.0 kpc out of the plane, but many fewer between 1 and 2 kpc.

Agreement dissolved over the issue of whether there is a third, nearly spherical, halo component. Neither radio nor X-ray data for our Galaxy seem to require such a component (S. L. Snowden, MPI für Extraterrestrische Physik, Munich), though they arguably do for some other spiral galaxies.

The theoretical point is that it is quite difficult for even very powerful expanding bubbles of hot, ionized gas, driven by many supernovae and stellar winds, to break out of the thick, ionized disk. The issue was addressed by José Franco (Univ. Nacional Autonomía de México), John Dyson (Univ. Manchester), Heinrich Völk (MPI Kernphysik, Heidelberg),

neutral gas, ionized gas at about 10^4 K (maintained by stellar photoionization and shocks), and coronal gas at 10^5 – 10^6 K (arising from supernova remnants and their mergers).

Cox, astoundingly, proposed that some of his own earlier conclusions had been wrong. He had previously suggested that the supernova remnants would sweep everything else out of the way, tearing up the diffuse material into hot gas bubbles with dense thin shells around them. As gas with an embedded magnetic field is rather incompressible, he now believes that the interface layers are thick and of low density contrast and that much of the material remains in smooth, diffuse phases. Elias Brinks (Royal Greenwich Observatory) showed H I maps of shell structures in the Andromeda galaxy (M31; see figure) supporting the latter model, whereas D. Breitschwerdt (MPI Kernphysik, Heidelberg) favoured the former one.

The stability and balance of the several phases ought, in principle, to be subject to

* IAU Colloquium No. 120 *Structure and Dynamics of the Interstellar Medium*, held in honour of Guido Münch, Granada, Spain, 17–21 April 1989.

calculation. Colin Norman (Space Telescope Science Institute) presented a simple analytical model in which the input parameters are the rate of mass and energy addition to the ISM and its clumpiness. It predicts that tightly wound spiral galaxies should have three stable ISM phases and more open ones only two. Other testable predictions include variations of the ISM components with radius and with time during a burst of star formation. The dense-molecular-cloud phase, which is dissipated once significant numbers of stars form, may be regenerated at the tips of expanding merged supernova-remnant bubbles, whose shapes have been distorted by the rotation of the galaxy, according to Jan Palous (Astronomical Institute, Prague).

Star formation, just alluded to, is obviously the single most important process in the life of a galaxy. It is also a very inefficient one, at least in the Milky Way, where typically only a few per cent of the mass in a giant molecular cloud complex has formed stars before the clouds are disrupted. We see no local examples of the kind of efficient star formation that occurs in starburst galaxies, according to Philip Solomon (State Univ. New York, Stony Brook).

Edith Falgarone (California Institute of Technology) pointed out that the known amount of turbulence in molecular clouds is sufficient to stabilize them against collapse and star formation on the free-fall timescale. Consistently, molecular clouds at the Galactic Centre, with larger turbulent velocity dispersions, are even less than averagely efficient star formers despite their high densities (Bally). One wonders, of course, why turbulence is less stabilizing in some other galaxies.

The low efficiency of star formation shows up in the total galactic rate at which gas is being turned into stars massive enough to ionize their surroundings and to give rise to core-collapse supernovae. Heiles, inventorying OB star associations and their adjacent ionized hydrogen gas, finds that a star bigger than 8 solar masses ($M_{\odot}$) — a likely minimum for type II supernova progenitors — forms only once every 112 years. Solomon, relying on a combination of carbon monoxide and infrared continuum emission, estimates that 0.5–0.6 $M_{\odot}$ per year goes into stars above 2 $M_{\odot}$ (ionizers), corresponding to about two core-collapse supernovae per century. The two estimates should be regarded as consistent within their errors. Both are at the low end of generally advertised rates for the Milky Way.

A second very important aspect of star formation is that it seems to be a two-stage process, according to Richard Larson (Yale Univ.) and Hans Zinnecker (MPI, Munich). A first, fragmentation, stage produces proto-stellar cores of characteristic mass a bit less than that of the Sun.

Origins of full-scale agriculture

THE harvesting of crops using replicas of ancient sickle-blades from the Near East, and comparison of the resulting wear on the replicas with that on the original blades, suggest that early soil tillage and plant cultivation began as long ago as the eleventh millennium BC. Romana Unger-Hamilton, now reports¹⁻³ her investigations of the lusted flint sickle-blades found commonly at some sites in the Near East.



R. Unger-Hamilton

Wild cereal on Mount Carmel.

She used almost 300 experimental flint blades of various kinds to harvest different species of the wild and cultivated plants common to the epipalaeolithic and neolithic sites of the Levant — wild progenitors of cereals (emmer, einkorn, barley), bread wheat and macaroni wheat, and plants growing among wild cereals, such as grasses, wild oats and vetches.

Unger-Hamilton finds that it takes about 10,000 strokes to develop a strong lustre on the blades, suggesting that the ancient blades were used for some time, perhaps as multi-purpose tools. The distribution of the polish on the blade depends on the species harvested, presumably because of the stem structure of the plant. The number of striations on the blade is also dependent on the type of ground in which the plant grows. The striations are caused by loose soil trapped at the base of the stems rather than by the plant itself. When the plants are cut close to the ground, the soil comes between

blade and stem, so the side of the blade turned towards the ground becomes more striated. This last observation supports the findings of Korobkova⁴, who first attributed microscopic striations on flint blades used to harvest crops to contact with soil loosened by tillage.

Unger-Hamilton's striations are particularly numerous on blades used to harvest macaroni wheat at Jericho, where weeds tend to trap the dust, compared with only one or two striations on blades used to harvest plants from a grassy cover, even after thousands of strokes at the base of the plants. These data can be compared with those on ancient flint blades from the southern Levant, dated to the natufian period (10,000–8,000 BC), and from Jericho in the early neolithic (8,000–6,000 BC). Only about a quarter of the natufian blades were heavily striated, compared with about half from the beginning of the early neolithic and three-quarters towards the end. Unger-Hamilton concludes that most of these blades were used to harvest cereal plants close to the ground, including the stem, during the early natufian, but that the degree of loose earth increased with time, indicating a change in harvesting from tilled soil. Although other factors may have been involved, Unger-Hamilton interprets her data to show that full-scale agriculture in the southern Levant began in the second phase of the early neolithic, about 7,000 BC, and that cultivation of cereals began in the early natufian, in the eleventh millennium BC. Paul G. Bahn

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These proceed to become garden-variety, low-mass stars. But many of the cores are surrounded by disks of varying mass and it is accretion from these that produces rarer, high-mass stars, in a power-law distribution ($N \propto M^{-2}M^{-2.5}$, typically). While the disks are present, the protostars have stronger gravitational (tidal) interactions than they will at later stages, and Larson suggested that this is likely to be important for understanding formation of binary stars, ejection of runaway stars from clusters, and other dynamical processes.

The dichotomy between high- and low-mass star formation shows up in several ways. Massive pre-main-sequence stars are more strongly clustered than low mass ones, according to separate infrared and radio mapping studies by C. Eiroa (Observatorio Astronomico-Ign., Madrid) and Eric Keto (Lawrence Livermore National Laboratory). The disks remain in evidence for about 10^6 yr around low-mass stars

(Anneila Sargent, California Institute of Technology) but only 10^4 yr around high mass ones (Keto). At the stage probed by Sargent's 1-mm continuum emission studies, the disks quite often have masses (0.001–0.1 $M_{\odot}$) and velocity structures (keplerian) much like the disk that is thought to have produced our own Solar System, suggesting that such planetary systems ought to be common.

Starting almost immediately after their formation, stars begin returning gas and energy to the ISM in winds, bipolar outflows and so forth, followed eventually by superwinds, planetary nebulae and supernova ejecta. But this is another comparably long story and will have to be told elsewhere. □

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Lost birds of New Caledonia

Colin Harrison

EVER since Darwin came away from the Galapagos Islands, island faunas have been seen as simplified microcosms of the complex webs of evolution and speciation evident from larger land masses. But islands are doubly interesting, because they may play host to highly specialized endemic species. There is increasing evidence that these faunas are not pristine examples of natural evolution, but have been substantially altered by human activity in the recent past to a previously unrecognized degree. This work throws doubt on the past use of extant island faunas in studies of zoogeography and speciation, and adds to the already irreparable damage done to some earlier concepts of noble savages living in harmony with their surroundings.

New Caledonia, in the South Pacific, is a case in point: the discovery of the bones of an enormous and previously unknown species of game bird, *Sylviornis neocaledonicae*, at first thought to be a relative of the emu, showed that at least one species endemic to the island has become extinct in the recent past. Jean Christophe Balouet and Storrs L. Olson (*Smithson. Contr. Zool.* **469**, 1–38; 1989) now add at least eleven extinct species of non-passerine bird to the tally of New Caledonia's recent dead. This represents a loss of one-third of the endemic non-passerine birds in recent times, although the final total might be closer to one-half.

Balouet and Olson record specimens of large megapodes, rails and pigeons related to extant species, as well as a larger sibling species of the kagu (*Rhynochetos jubatus*), an extant endemic species of rail. The fact that these birds were mostly larger species may be significant; their disappearance appears to have coincided with the appearance of man on the island less than two thousand years ago. When human hunting occurs, larger species tend to disappear before smaller ones. The large species on New Caledonia were presumably lost at the hands of man.

An ever-increasing body of evidence from around the world tells the same story. We now know that the human settlement of Hawaii, for example, resulted in the loss of half the endemic bird life; the faunas of the larger islands of New Zealand and Malagasy were devastated by man; and smaller oceanic islands show similar evidence of extinctions associated with human occupation. Although most of the new data come from studies of Indian and Pacific ocean sites, significant species losses also occurred on Atlantic, Caribbean and Mediterranean islands in the recent past.

This evidence of recent destruction

throws doubt on evolutionary inferences based on the assumption that extant island faunas represent complete, untouched ecosystems. Island faunas in their native state were larger and more diverse than has been assumed, and often contained a range of surprisingly similar species, such as the kagu and its extinct, larger sibling.

Zoogeographical inferences, drawn from the patterns of distribution of taxa

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

The dodo (*Raphus calcullatus*) from Mauritius and nearby islands in the Indian Ocean was perhaps the best known casualty of extinctions on islands attributable to human beings. (Courtesy British Museum (Natural History)).

among islands, are also called into question because the new data show that these patterns are sometimes incomplete. In other cases, the loss of endemic species on islands may have allowed opportunistic species of colonists to invade and establish themselves. The resulting distributions are more extensive than would have been possible, were the endemic competitors of invading species not previously driven to extinction by human action.

Extinction of species through human activity therefore creates a dynamic situation, involving sequences of both loss and gain. New Zealand is often cited as an example of the problems resulting from unwise introductions of species, but this may in fact be true only for islands that have previously been the victims of extensive impoverishment of species and modification of habitats.

It is clear that the outcome of these new studies of fossil remains on islands will not only be to present a picture of a greater and more strikingly varied bird fauna, subsequently impoverished by human activity, but also will necessitate reconsideration of the ideas and theories that have been based on present island faunas. □

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Breath of life

PEOPLE are absurdly selective about their food. Each culture has a limited range of acceptable foodstuffs, and rejects all others as distasteful or offensive. With the world population growing faster than ever before, we can no longer afford this fastidiousness. So Daedalus is inventing the ultimate in cheap, tasteless, anonymous junk food.

DREADCO's biochemists are pre-digesting all sorts of plant and animal products by modern chemical and enzymatic hydrolysis methods. All the sugars, amino acids and fatty acids of biological digestion are generated as a clear solution which you could simply drink. But Daedalus has other ideas. He recalls a cunning new fire-fighting system which saturates the air around the fire with a dense fog of water droplets. This water-laden air can still be breathed in a sodden sort of way. So, says Daedalus, a similar fog of hydrolysed food-solution could also be breathed. The breather would absorb its nutrients directly: not through his stomach, but his lungs.

The DREADCO workers are accordingly inventing a sort of 'personal carburettor' whose spray nozzle fits on a spectacle frame and injects the food-mist into the wearer's nostrils with every breath. At its most elegant, the device could be coupled to an expired-carbon-dioxide meter or a heart-rate monitor, to register the wearer's metabolic rate from moment to moment and provide him with exactly the right energy intake. Absolute metabolic balance would be guaranteed: the wearer could never gain weight. But a less obtrusive variant, DREADCO's 'nocturnal carburettor', will have wider appeal. Fastened to the bed-frame and feeding the sleeper's nostril injector via flexible pipes, it provides him with his daily nutritive requirements during the hours of darkness.

Thus the world's food resources will be harnessed to the full. DREADCO's hydrolysis process will not merely accept approved cereal grains and selected animal muscles; stalks, weeds, leaves of all sorts, whole animals and pests of every kind, offal and waste and general organic junk, all will be grist to its mill. The sterile hydrolysate will be filtered from bones, and so on, and optimized to human needs (amino-acid ratios may need adjusting; various trace vitamins and minerals may have to be added).

A dedicated eager beaver could live indefinitely by nocturnal carburetting, devoting his waking life entirely to work or socializing, and would never need to waste time in eating at all. But the best approach is probably to gain most of one's needs through the carburettor, and eat such foods as one's culture approves in a refined and delicate gourmandizing, merely for enjoyment and the exercise of the alimentary tract.

David Jones

Measurement of γ -rays from cold fusion

SIR—Petrasso *et al.*¹ have recently published a critique of the γ -ray spectrum given by three of us² as supporting evidence for the solid-state fusion of deuterons in palladium host lattices. The basis of this critique was the nature of a γ -ray spectrum displayed during a television broadcast. One of us (M.H.) denies the accuracy of ref. 6 of Petrasso *et al.*; M.H. did not state that the quoted television spectrum was made in these laboratories, as it most certainly was not. In view of this somewhat strange approach to the collection of scientific data and, as we cannot vouch for the authenticity of the spectrum transmitted (we have now confirmed that the "curious structure" in the television 'data' given by Petrasso *et al.* — their Fig. 1b and legend¹ — is simply the trace of a screen cursor on the multi-channel analyser visual display unit!), we give in the first place one of the complete set of spectra recorded at that time (Fig. 1).

In the work reported by us², γ -ray spectra were measured principally to check on the safety of our operations and, as we have repeatedly pointed out, we are well aware of the deficiencies of these spectra. Figure 1 gives the background spectrum ('sink'; solid line) taken over a sink containing identical shielding

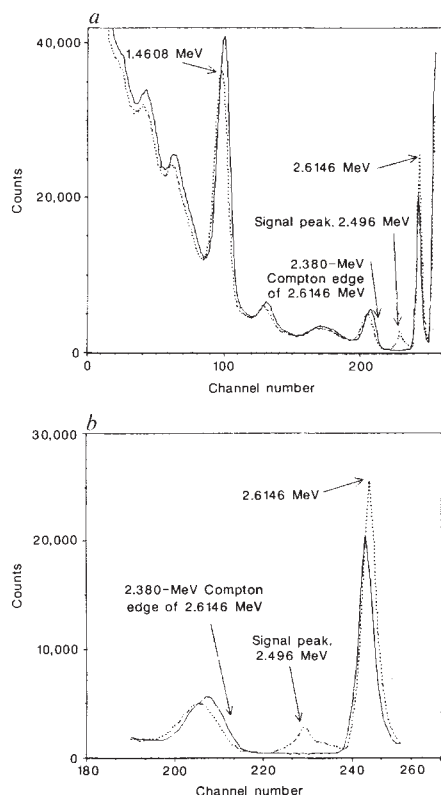


FIG. 1 The γ -ray spectrum accumulated over the water bath containing the electrolytic cells ('tank'; dotted line) and over a sink 5 m away ('sink'; solid line). Detector is a 3 $\times$ 3 in. NaI right circular cylinder. Spectral accumulation times: 50 h. b is an expanded version of the most relevant region of a.

materials but at a distance of 5 m from the tank containing the experimental cell. This cell contained a 0.4 $\times$ 10 cm palladium electrode polarized at a current density of 64 mA cm⁻²; during the period of the measurement it was generating excess heat at the rate of 1.7–1.8 W (over and above that due to the electrode reactions).

The 'peak' under discussion is centred at 2.496 MeV, and it can be seen in Fig. 1a that all the peaks in the background spectrum on the low-energy side of 2.496 MeV are displaced to higher energies whereas that on the high-energy side (due to ²⁰⁸Tl) is displaced to a lower energy. (Scaling of the spectrum made near the electrolytic cell with a quadratic interpolation formula generated the spectrum we reported: this scaling produced a shift and narrowing of the 2.496-MeV peak.) The observed shifts are due to a combination of zero shift in the analyser and a gain shift of the NaI detector resulting from drift of the pre-amplifier. Over the long data-acquisition times, the shifts are of little importance. The spectra do indicate, however, that the nature of the background radiation in the two areas of the laboratory is essentially the same. The only significant difference between the spectra is the signal peak. This is very convincing evidence that the signal peak is not due to products of radon decay.

It can be seen, however, that there is another unexplained feature in these spectra: there is a rising tail at the end of the spectra. This is due to pulse pile-up in the last few channels as a result of a peak at slightly higher energy than the 2.6146-MeV peak (Fig. 2). Figure 2 represents a background spectrum that was acquired with a slightly reduced gain so that the energy window could be extended.

The exact interpretation of the 2.496-MeV peak is in doubt; certainly, the peak from the reaction $^1\text{H} + n \rightarrow ^2\text{D} + \gamma$ (2.22 MeV) would be expected to lie to the left of the Compton peak that arises from the thorium decay chain. The search for this peak does not seem to be feasible using NaI detectors. In spite of the problems underlying the interpretation of these spectra, we consider that the measurements show the emission of γ -rays from the cell environment: removal of the cells leads to the removal of the signal peak. A possible interpretation is that the signal peak is a single- or double-escape peak from 3.01- or 3.52-MeV peaks, or from summing of other unidentified peaks at lower energies. The unusual shape of the signal peak suggests that it may be a combination of such peaks. The size and energy of the signal peak imply that any associated Compton edge or escape peak will be lost beneath the rest of the spectrum.

Petrasso *et al.*¹ have also commented on

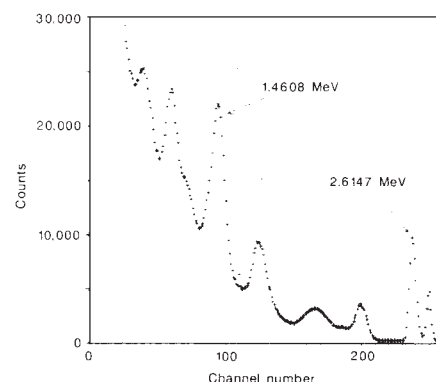


FIG. 2 The γ -ray spectrum accumulated in a similar manner to those in Fig. 1 in a remote laboratory at reduced gain.

the integrated peak intensity of the γ -ray spectrum reported by us and imply that we sought to relate this to the neutron count observed close to a similar cell operated in the open air. We point out that we made no such comparison but instead sought to relate the neutron count rate to the tritium production which we and others have observed. Clearly, further work on the γ -ray spectra should include the characterization at high resolution with solid-state intrinsic germanium detectors of the γ -ray emissions in the energy region above 2 MeV.

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PETRASSO *ET AL.* REPLY—Our criticism¹ of the published 2.22-MeV neutron-capture-on-hydrogen γ -ray line of Fleischmann *et al.*², claimed by them as compelling evidence of neutron production in their electrochemical cells (Fig. 1a of erratum of ref. 2; Fig. 2 of ref. 1; our Fig. 1 here), raised two fundamental points: Fleischmann *et al.*'s γ -ray line first is a factor of two narrower than their instrumental resolution would allow, and, second, lacks a Compton edge, which should be distinctly evident at 1.99 MeV (Fig. 1). We therefore concluded that their γ -ray signal line was an instrumental artefact, and we argued that the energy position of their signal line was unlikely to be at 2.22 MeV as they claimed. We suggested that the energy of the signal line could easily be verified by publication of their full γ -ray spectrum, because prominent, naturally occurring background lines from ⁴⁰K (1.46 MeV) and ²⁰⁸Tl (2.61 MeV) calibrate their spectra absolutely^{1,3,4}.

In their response above, Fleischmann

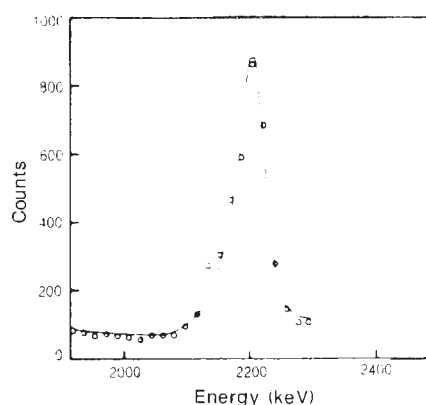


FIG. 1 A reproduction of the purported 2.22-MeV neutron-capture-on-hydrogen γ -ray line of Fleischmann *et al.* (Fig. 1a of ref. 2, erratum). As we discuss in ref. 1, the resolution of their NaI spectrometer would be about 2.5% based on this linewidth. With such resolution, one would expect to see a clearly defined Compton edge at 1.99 MeV. No edge is evident. Also, a resolution of 2.5% is inconsistent with their spectral resolution (Table 1b of ref. 1). Because of these inconsistencies, we argue that this signal is an instrumental artefact¹. In this figure, Fleischmann *et al.* show the peak energy to be at 2.22 MeV. In their response above, they indicate that their γ -ray signal line has an energy of 2.496 MeV (see peak 7 of Fig. 2a and text).

et al. fail to address our key criticisms concerning their published 2.22-MeV γ -ray line. They further claim, erroneously, that their televised γ -ray spectrum¹ was the basis of our analysis. (Our quantitative analysis is based on their published signal line (Fig. 1), their instrumental resolution (Table 1b of ref. 1), a controlled neutron experiment (Fig. 3 of ref. 1) and the well-known properties of NaI scintillation detectors^{5,6}.) Fleischmann *et al.* do, however, show their full γ -ray spectra (our Fig. 2a), in which they identify a signal line to have an energy not of 2.22 MeV, but now of 2.496 MeV. (No explanation is given for their original identification of 2.22 MeV (Fig. 1).) They contend nonetheless that this signal line is proof of true γ -ray emissions from their heat-producing cell, although unrelated to the 2.22-MeV neutron-capture γ -ray. Unfortunately they are unable to identify the nuclear process that generates this 2.496-MeV γ -ray, or to account for its distinctly unphysical lineshape. We again suggest that their signal line is an instrumental artefact unrelated to a γ -ray interaction — this holds both for their signal line in the errata of ref. 2 and for that in their response above (peak 7 of our Fig. 2a).

In regard to their γ -ray spectra (Fig. 2a), we argue that Fleischmann *et al.* have misidentified the ^{208}Tl peak and therefore that their energy calibration and interpretations are correspondingly suspect. We have numbered the peaks in their spectra so as to best match our energy identifications^{1,3,4}, and we show beneath their γ -ray

spectra one obtained at MIT with a 3×3 in. NaI(Tl) crystal (Fig. 2b)^{1,7}. (See refs 1 and 2 for a discussion of the 3×3 in. NaI detector used by Fleischmann *et al.*².) The energy scales in Fig. 2 have been scaled and aligned so that the ^{40}K and (true) ^{208}Tl peaks (peaks 3 and 6, respectively) coincide for both spectra. There is a clear correspondence between the Fleischmann *et al.* and MIT spectra for all energies below the (true) ^{208}Tl peak at 2.61 MeV. To further test our energy identifications for the Fleischmann *et al.* spectrum, we plot in Fig. 3 their channel number against our line-energy identifications. The relationship is approximately linear, as it should be if our identifications are sensible. (For the weaker 'peaks' (1, 2, 4 and 5) the precise centroid of energy is a function of the local terrestrial radiation environment⁴.) Based on this calibration curve (Fig. 3), their purported γ -ray signal line resides at about 2.8 MeV, not at 2.496 MeV.

The next issue is to identify the other features in the spectra of Fleischmann *et al.* (peaks 7, 8 and 9 in Fig. 2a) above the (true) ^{208}Tl line (peak 6). Peak 8, very prominent in both background (sink; solid line) and the cell (tank; dashed line) experiments, and identified by Fleischmann *et al.* as the ^{208}Tl peak, is unphysical, as its linewidth is more than two times smaller than their instrumental resolution would allow (based on the ^{40}K linewidth in Fig. 2a; see also refs 1, 6, 7). This is exactly the same objection that we raised in regard to their purported 2.22-MeV γ -ray line (Fig. 1). This observation especially reinforces our contention that peak 6, rather than 8, is to be identified with the ^{208}Tl line. A similar criticism of the linewidth can be made of peak 9 (see Fig. 2 of Fleischmann *et al.* above). The unphysical shape of peak 7, their signal line, also argues against its identification as a real γ -ray line. Thus we conclude that all ' γ -ray signals' above the (true) ^{208}Tl peak (6) are instrumental artefacts. We further point out that in our background spectra (Fig. 2b) and in the standard works on γ -ray background radiation^{3,4}, there is no evidence for any strong lines above the (true) ^{208}Tl peak. Therefore, Fleischmann *et al.*'s present claim of having observed γ -ray emissions from their cell is unfounded.

Another important issue is whether 2.22-MeV neutron-capture γ -rays actually emanate from the water bath surrounding their heat-producing cell which, according to ref. 2, generates 1.7–1.8 W. Using our energy calibration for their spectral data (Fig. 3), and looking in the vicinity of peak 5 of Fig. 2a (which is close to 2.22 MeV), we find no evidence for even a small change in the background spectrum relative to that of their heat-producing cell. Quantitatively, from our controlled neutron experiment in a water bath^{1,7} and the spectral data of Fig. 2a, we can estimate an upper limit on the neutron production rate

of about 4×10^2 neutrons s^{-1} . This bound is a factor of 100 smaller than the rate Fleischmann *et al.* claim to have actually observed with their neutron detector². (Fleischmann *et al.* purport above that NaI scintillation detection is inadequate to detect 2.22-MeV neutron-capture γ -rays. This view is erroneous^{1,5,6}.)

In conclusion, Fleischmann *et al.* fail to answer our key criticisms¹ of their published γ -ray spectrum², in particular the spurious nature of their purported 2.22-MeV neutron-capture γ -ray line (our Fig. 1). Although they inexplicably no longer claim to have observed the 2.22-MeV neutron-capture γ -ray, they now

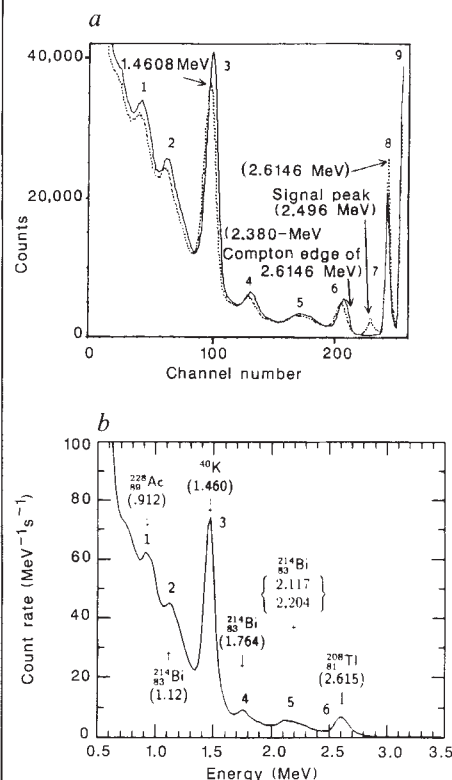


FIG. 2 The γ -ray spectra from a, the Fleischmann *et al.* experiments (solid and dotted lines are for 'sink' and 'tank', respectively), and b, the MIT γ -ray background measurements. In these two figures, the various peaks have been identified with numbers. (Peaks 1, 2, 4 and 5 are actually blendings of many lines^{3,4} of which we have identified the main contributors.) In a, Fleischmann *et al.* have identified the ^{208}Tl line with peak 8; we believe that it should instead be peak 6. Consequently we have placed in parentheses their energy identifications and remarks which we believe are in error, and we have accordingly recalibrated their data. With this recalibration, a and b have been drawn on the same energy scale. Fleischmann *et al.* identify peak 7 as their γ -ray signal line, that is, as the indicator of actual nuclear γ -rays emanating from their heat-producing cell. They now identify its energy as 2.496 MeV, not 2.22 MeV as in their earlier work². (See also Fig. 1.) Using our energy calibration (Fig. 3), peak 7 corresponds to an energy of about 2.8 MeV. We argue that the Fleischmann *et al.* peaks of 7, 8 and 9 are instrumental artefacts; see text.

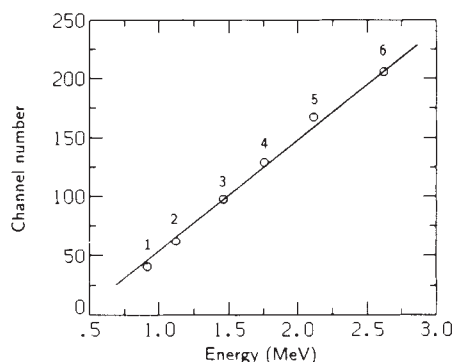


FIG. 3 The energy calibration for the γ -ray spectrum of Fleischmann *et al.* (tank spectrum, dotted line of Fig. 2a) using the energy identifications of Fig. 2b. Note that we have identified the ^{208}Tl line (2.61 MeV) with peak 6, not 8 as Fleischmann *et al.* did. The straight line is drawn through the ^{40}K (1.46 MeV) and the ^{208}Tl (2.61 MeV) points, which are the most prominent background lines^{3,4}.

contend that their γ -ray signal line is a true γ -ray of energy 2.496 MeV and, most importantly, that this signal is evidence for nuclear reactions in their cell. They make this claim despite their inability to identify the nuclear process associated with their purported 2.496-MeV γ -ray or to account for its distinctly unphysical lineshape. Furthermore, after correcting their spectral data for a miscalibration in energy, we argue that their signal line (with a correct energy of 2.8 MeV rather than 2.496 MeV) as well as all lines above their (true) ^{208}Tl peak (2.61 MeV; peak 6) are instrumental artefacts in the upper channels of their spectrum analyser. Finally, using our calibration for their γ -ray spectra (Fig. 3), we find no evidence for the emission of 2.22-MeV neutron-capture-on-hydrogen γ -rays from the water bath surrounding their heat-producing cell. Quantitatively this can be interpreted as imposing an upper bound of 4×10^2 neutrons s^{-1} for the production of neutrons. This limit is a factor of 100 times smaller than the neutron rate Fleischmann *et al.* claim to have actually observed².

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Fast pulsations in supernova 1987A

SIR—The extremely fast pulsations reported in the 2-year-old supernova SN1987A in the Large Magellanic Cloud¹ will pose serious problems if the pulsations are caused by stellar rotation². I would like to suggest an alternative model where the fundamental mode of stellar radial vibrations is responsible for the short period.

In a supernova explosion some of the matter near the centre which was ejected earlier but with less than the escape velocity should fall back to the star. I assume that such accretion of the infalling ejecta is taking place near the Eddington limit and that the inner region of the accretion flow is in the two-temperature phase with the corresponding equipartition magnetic field, B , of about 10^7 – 10^8 gauss³.

In magnetized accretion flows of this type electron cyclotron radiation is emitted³; because the cyclotron optical depth for the fundamental and higher mode harmonics is larger than one, these photons are self-absorbed up to the critical frequency where the optical depth becomes one³. The flux of this self-absorbed cyclotron emission can be calculated from the equation derived in ref. 3.

Adopting as the typical physical parameters the accretion rate $\dot{M} = 10^{-8} M_{\odot}$ per year, the stellar mass $M = 1.4 M_{\odot}$, the electron temperature $T_e = 2 \times 10^9$ kelvin, and $B = 10^7$ gauss in the inner part of the accretion flow at a distance of $10 \times$ the stellar radius, we found the total cyclotron flux emitted over the optical region to be $F_c = 8.4 \times 10^{18}$ erg cm^{-2} s^{-1} . The corresponding total luminosity is 2.7×10^{35} erg s^{-1} , which agrees with the observed optical pulsar radiation.

The flux depends sensitively on the magnetic field strength, B , and very small periodic variations of B near the surface caused by the stellar radial vibrations can, through shocks, produce observed periodic variations in F_c . The light-travel time at a distance of 10 stellar radii, where most optical pulsar radiation is emitted, is less than the pulsar period, and thus the pulsed radiation will not be washed out. The sharp pulses can be maintained as the infalling clouds are expected to be highly inhomogeneous. A decrease of B by a factor of only 2 will reduce the total luminosity to 2×10^{33} erg s^{-1} , fainter than 20 mag. Therefore, in my model, the reported disappearance of the pulsar to below 20 mag can be explained by a small decrease of B .

My model is fundamentally different from the vibration-cyclotron model proposed by Wang *et al.*² in the following senses. First, whereas I assume the accretion near the Eddington limit is the energy source, Wang *et al.* assume the energy source is the residual vibration. Second,

I adopt a weak field model whereas Wang *et al.* require B to be about 10^{12} gauss. Finally, for Wang *et al.* the optical pulsed emission is caused by the ion cyclotron fundamental mode emitted right on the surface, while in my model it results from the self-absorbed electron cyclotron higher harmonics emitted within the accretion flow, near to, but above, the surface.

Observational tests should be able to distinguish between the two models. If, within a few years, the GINGA X-ray satellite detects X-rays directly emanating from the hot neutron star accreting near the Eddington limit, this should support our model and exclude that of Wang *et al.*. Also, if slower rotation-powered pulsed radiation is detected in SN1987A, the exact value of the period should distinguish between the models.

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Taxonomy debate signing off

SIR—In response to A.S. Clare and D. Rittschof (*Nature* **338**, 627; 1989) I would like to point out that trinomia are already used in taxonomy, but that the name in brackets is reserved for the sub-genus.

Also, the equals sign means synonymy, but a species being moved from one genus to another does not mean that the previous genus is synonymized unless the species happens to be the type species. To be correct, it should be *Semibalanus balanoides* (= *Balanus balanoides*), assuming that the species has not been split up during transfer.

Clare and Rittschof might consider quoting the species name in full by adding its author and date. If the species has been transferred then the original author and date go in parenthesis as is the norm and, borrowing from botany, the name and date of the latest revisor can be added after the original author. The taxonomic history of the species should now be traceable.

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This correspondence is now closed — Editor, *Nature*.

Seal disease predictions

SIR—It is now generally accepted that the primary agent responsible for the deaths of more than 17,000 common seals (*Phoca vitulina*) in Europe since April 1988 is a previously undescribed morbillivirus, phocine distemper virus (PDV)¹. At least 185 grey seals (*Halichoerus grypus*) have been found dead around the United Kingdom during the same period, some with pathological symptoms similar to those of common seals. At present we do not know enough about the virus or the behaviour of seals to model its epidemiology realistically but there is sufficient information to make some broad predictions.

The speed with which the disease has spread across Europe, and the fact that seals periodically form dense aggregations on sandbanks or rocks to pup and to

moult, suggest that the prevalence of the virus within a local seal population should reach high levels within months of first exposure. This prediction was confirmed when we tested samples of blood from adult grey seals and their pups collected at three colonies in Scotland in October and November 1988. The presence of serum antibodies against canine distemper virus (CDV), which is closely related to PDV, was tested by a virus neutralization assay and with an ELISA test using CDV proteins as the antigenic target; results from these assays are highly correlated². All but 2 of the 73 sera contained antibodies to CDV but no antibodies were detected in sera taken from grey seals between 1977 and 1987, including at least five animals that were subsequently resampled in 1988. These results confirm previous suggestions that PDV was introduced into the seal populations of the North Sea during 1988, although they provide little insight into the origin of the virus.

When we tested blood samples from surviving and apparently unaffected common seals collected at a number of UK sites in 1989, as part of a project funded by the British Department of the Environment, we were surprised to find that only half had a significant immune response. As shown in the table, however, most of the older (> 2 yr) animals had serum antibodies whereas few of the younger ones did. This implies that many of the younger seals have yet to be exposed to the virus.

We would expect approximately one third of a seal population with a stable age structure to be in age-classes 0 and 1 eight months after the pupping season, when our samples were taken. Provided there has been no differential mortality among the age-classes (and there is no conclusive

evidence of this³) we can calculate approximate 95 per cent confidence limits for the proportion of susceptible animals in the surviving population using a normal approximation 0.285–0.465.

Thus between about one quarter and one half of the surviving British common seal population has yet to develop an immune response to PDV and could be susceptible to infection with PDV in 1989. These animals are concentrated in the youngest age-classes. Their susceptibility to fatal infection may be increased by the apparently poor immune status of young common seals². They will be particularly vulnerable to infection during the moult in August when large and dense aggregations of all age-classes form, except pups born that year. If there are still infected animals around at this time, we predict that there could be substantial mortality among the youngest age-classes.

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Antibodies against CDV in common seals sampled in 1989			
Site	Age* (yr) tested	No. tested	Antibody- positive(%)
The Wash	<2	3	0
	>2	3	100
Dornoch Firth	<2	10	20
	>2	6	67
Orkney	<2	4	25
	>2	12	100
Mull	<2	2	0
	>2	4	50
Strangford Lough	<2	5	70
	>2	7	100
Total	all	56	55
	<2	24	12
	>2	32	88

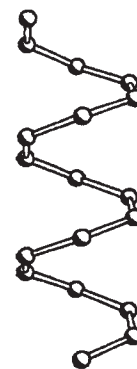
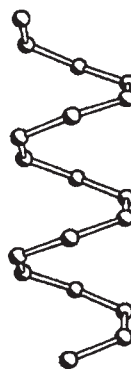
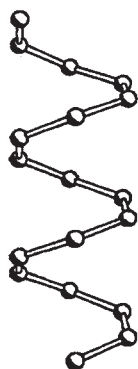
*Seals were divided into age classes on the basis of length; those less than 110 cm were considered to have been born in 1988 or 1987.

Stereopsis ambiguity in stereo images

SIR—Tucker¹ is right to ask those who publish stereo pairs to mention for which eye each element of the pair is intended. In many cases, however, the potential ambiguity can be removed by various techniques.

The helical structure shown here, for example, can be viewed in only one possible handedness because of the care taken to represent volumes.

Thus, the three-dimensional image of the right-hand pair viewed by direct stereopsis exhibits some contradictory



details, indicating it is the incorrect orientation.

Many programs are available that are able to remove the stereopsis ambiguity. Perhaps the most popular is ORTEP², in which each stereo pair can be unambigu-

ously identified when properly treated.

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1. Tucker, V. *Nature* **337**, 605 (1989).

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The great egg debate

A. A. Glynn

Safe Shopping, Safe Cooking, Safe Eating: Simple Rules That Can Protect You and Your Family. By Richard Lacey. Penguin: 1989. Pp. 177. £2.99.

Salmonella in Eggs. Vol. I Report and Proceedings of the Committee. Vol. II Minutes of Evidence and Appendices. Agriculture Committee of the House of Commons. HMSO: 1989. Vol. I pp. 31, £4.70. Vol. II pp. 340, £15.40.

WHERE food is concerned everybody is a compulsory, though usually willing, consumer. In the developed world it is a long time since everyone, or even every family, was also a producer. Food production and processing are now huge industries and have their faults, as Richard Lacey of the University of Leeds is keen to point out, but without such specialization it is difficult to see how present-day populations could be adequately, let alone safely and agreeably, fed. Moreover the current Western increase in food-borne infections is insignificant compared to those rife in the underdeveloped world. A sense of perspective should not of course stop us looking at our own immediate difficulties, but that the public as a whole is looking at them is largely an accident of media hyperbole.

Food-borne infections due to bacteria of the genus *Salmonella* have been increasing in frequency for many years. Overall the predominant species has been *S. typhimurium*, but from time to time others have come into prominence for a year or two and then faded away. For example, *S. hadar* (from turkeys) was particularly frequent for a few years in the 1970s but then decreased as the turkey industry cleaned itself up. For a short time *S. hadar* lay second to *S. typhimurium* but never overtook it, a feat achieved in Britain by *S. enteritidis* in 1988.

Reported isolations of *S. enteritidis* in the north-east of the United States rose sixfold between 1976 and 1986. Their relation to eggs was first reported in April 1987. In Britain the rise started later. Isolations of *S. enteritidis* reported to the Public Health Laboratory Service (PHLS) rose sixfold between 1981 and 1987, and actually doubled in 1988. Two particular strains of *S. enteritidis* seem to be involved, phage type 8 in the United States and phage type 4 in Britain. There is evidence that both may get into the egg before it is laid, via the oviducts of the infected chicken. Such a route was described in 1930 and has always been important in duck eggs. With hens' eggs the more usual route of infection, at least up to now, has been by fecal contamination of the shell after laying.

Although the Department of Health issued five separate warnings in the second half of 1988, they caused little stir. Only on 3 December 1988, when a junior minister of health, Mrs Edwina Currie, made her now notorious statement that "most of the egg production of this country, sadly, is now infected with salmonella. . .", did journalists turn their whole attention on the problem. Egg sales fell disastrously, at least for a time, and the incident achieved what public health

preservation and food poisoning. Much of the information is standard and the advice on the whole conforms to generally accepted methods and precautions. The concepts of infective dose and of healthy carriers are well explained. Lacey's special interests and prejudices are apparent, as he himself recognizes when he stresses a point by saying "this is not just the author's whim". Although this makes for livelier reading, those unfamiliar with the field may be excused the impression that, where food poisoning is concerned, Leeds is the centre of information, and virtue.

It is difficult to write popular science which is also acceptable to the experts and Lacey does not. To say of listeriosis that "All the cases in the world with a proven source other than food probably add up to less than ten" is misleading when the source of most cases is unknown and certainly unproven. Some of the case histories seem to be included more to

produce emotion than enlightenment. The 'typical' history of a still birth due to *Listeria* is only typical in as much as the source of the infection was unknown.

In the second part of the book, Lacey uses an amusing and informative review of the food shelves in a supermarket to bring out possible problems and how to avoid them. Restaurants get tougher treatment, though Lacey gets carried away when he describes what to do if served with foul-smelling meat in an advanced stage of decomposition, thereby spoiling the thesis that most infected food appears to be perfectly normal. He also appears to have a nostalgia for a less-technological culinary past which detracts from the effort to make the future

better. Thus he prefers canning to deep freezing, has little use for cook chilling or irradiation, and not much for additives except, surprisingly, nitrites.

Although canning is one of the safest ways of preserving food, inadequate heating, or later contamination of a damaged or improperly sealed tin, can give rise to one of the most deadly forms of food poisoning, botulism, due to growth and toxin formation by the anaerobic bacterium *Clostridium botulinum*. One has to hand it to Lacey. His warnings of the risks of food poisoning have been dramatically emphasized by the current outbreak of botulism in Britain, the vehicle of spread in this case being hazelnut yoghurt. The number of cases, over 20 so far, is more than the total number reported in Britain in the past 60-70 years. That is great cause for concern; but that only one elderly lady (at the time of writing) has died in the outbreak is a testimony to the value of the

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Humpty Dumpty's fall — the same happened to the British egg industry last December. (Mary Evans.)

workers had previously failed to do — it at last forced public and politicians to realize that they needed to think, or perhaps even do something, about food poisoning.

The politicians were perhaps readier to look at the problem because of a serious outbreak early in 1988 due to *S. typhimurium* in mayonnaise. The outbreak, coyly described in the *Lancet* as "in a large metropolitan building", was actually in the House of Lords and must have embarrassed the government in more ways than one. Further action is promised, but meanwhile we have a book, *Safe Shopping, Safe Cooking, Safe Eating*, and the report of a parliamentary select committee, both of which were inspired by the same events but which are very different in aims and style.

Safe Shopping is a very personal book aimed at those who buy and prepare their own food (that is, all of us at one time or another). The first part gives a broad and necessarily simplified account of food

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Edwina Currie (dubbed "Eggwina" by the tabloid press), the politician at the centre of the *Salmonella* scare.

high-technology medicine so frequently criticized.

Like everyone, Lacey has to face, but cannot resolve, the dilemma that fewer preservatives mean shorter shelf lives. Food technology has been improving since ancient Egypt or earlier and it is always the most recent innovations that are most criticized, not always rightly. Mège-Mouriès who patented the production of margarine in 1869 was later attacked by l'Académie des Sciences worried about the effects of the fatty acids in it.

Public health professionals have long known that food poisoning was increasing and that published figures seriously understate the real prevalence. To estimate the latter Lacey uses a multiplier of ten (the figure used in the United States is 100). Scientifically it would be preferable to argue from the basic published figures but to remember the multiplier when it comes to their application. For some reason Lacey assumes that the US figures for *Listeria* morbidity and mortality are complete. Yet proportional to the population they are little different from the British figures which he wants to multiply by an arbitrary three. It is interesting that the select committee, who as politicians are keenly aware of the impact of food poisoning, also criticize the use of multipliers.

A recurring theme both in the book and the report is the lack of reliable information. Although this is sometimes a reflection of a failure to look for it, compounded by erratic speculation in the press, it is clear that regular reliable official figures would be very useful. In the United States, such figures are available in the *Mortality & Morbidity Weekly Report* (MMWR). The British equivalent is the weekly *Communicable Disease Report* (CDR) frequently referred to by the media as a "secret government report". It is difficult to see how a document which goes out weekly, albeit confidentially, to some 5,000 people can seriously be regarded as secret. Journalists and others

obviously have no difficulty in quoting from it. But the parliamentary report rightly recommends that the question of CDR's confidentiality should be reconsidered.

The report has a much narrower remit than *Safe Shopping*. It deals clearly and comprehensively with the risks from *Salmonella* in eggs and the effectiveness of the government's response. It concludes that the risk is real, though slight for people in normal health, but that the response to last year's events was sluggish and inadequate. The written and oral evidence, given in full, is a useful source of reference, ranging from the carefully analysed graphs and tables provided by the PHLS, through closely argued cases put forward by associations with special interests, to strongly felt letters from individuals. The richest sections are those where the select committee grills its witnesses, whether they be acknowledged or self-appointed experts, civil servants or ministers. Parliamentary select committees in Britain are new compared with their congressional equivalents in the United States, but are learning fast. It is fascinating to compare how politicians, civil servants, scientists and others present their case and deal with evidence, especially when the conclusions are unwelcome.

The report criticizes the various Acts and Orders which are meant to control the food industry and the way they are monitored. Britain is one of only three countries which have taken advantage of an EEC rule allowing primary food industries to be exempted from consumer protection acts. But even if regulations governing the food industry are greatly strengthened, J. K.

Galbraith's concept of countervailing power suggests that more improvement may come from the consumer-conscious large retail chains exercising what one select committee member called "their considerable clout".

Montaigne, who complained that cooks discuss problems of food as seriously as major points of theology and in words appropriate to affairs of state, would have appreciated Lacey's book. Lewis Carroll would have preferred the report, whether it was dealing with Mrs Currie's evidence, ("is this an inquiry into what I said. . .?") or deciding what constitutes an epidemic. Mr Curry [sic] of the select committee queried the frequent use of the word in the PHLS report, backing his challenge with conflicting definitions from several dictionaries. However Joe Smith (PHLS) stood his ground and hit back with the shorter Oxford and a technical definition. On appeal Acheson (Department of Health) as a professional epidemiologist had no doubt the word was rightly used but advised against it as it might produce "irrational responses". Meldrum (Ministry of Agriculture), speaking for chickens rather than people, was clear that there had been an increase in incidence of infection but was not calling it an epidemic. He did not say if he called it an epizootic.

"When I use a word" said Humpty Dumpty, "it means what I want it to mean". But presumably pasteurized by now, he was not available for further comment. □

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Sunny planet

David Morrison

Mercury. Edited by Faith Vilas, Clark R. Chapman and Mildred Shapley Matthews. University of Arizona Press: 1988. Pp.794. \$45, £35.

EXTREME examples test and often illuminate broader truths. In planetary science, Mercury, Pluto and Jupiter all represent 'end-member' planets — objects that stretch the limits of our understanding of what constitutes a planet, and hence the conditions and processes that gave rise to the Solar System some 4,500 million years ago.

Mercury and Pluto are also the two smallest planets and the most difficult to study: Pluto because of its great distance from Earth, Mercury because of its proximity to the Sun. Mercury is never separated from the Sun by more than 27 degrees, so that it cannot be studied telescopically against a dark sky. In addition, its location deep within the gravity field of

the Sun makes it a difficult target for spacecraft investigation. Mariner 10 is the only craft to have reached Mercury, executing three exploratory fly-bys in 1974 and 1975. Little wonder, then, that this remarkable planet has been neglected in the rush of new discoveries that has revolutionized our understanding of the Solar System over the past quarter of a century.

As an end-member planet, Mercury has much to tell us. It is subject to the most intense solar radiation, and experiences the greatest diurnal temperature variations (although it does not have the hottest surface, that distinction belonging to Venus). It is the only planet whose rotation is tidally locked to the Sun. Mercury also has the most extreme composition, being made up largely of iron and other metals at the expense of silicate rocks as well as the more volatile elements. George Wetherill of the Carnegie Institute of Washington has recently argued (in this book and elsewhere) that the loss of much of Mercury's rocky mantle is the consequence of a catastrophic impact early in the history of

the Solar System, in which case this planet also represents the extreme case of planetary modification by collision — a collision that only just fell short of complete fragmentation.

The purpose of the book reviewed here is to revive interest in Mercury, more than a decade after the Mariner 10 fly-bys. Like other volumes in the highly successful University of Arizona Press *Space Science* series, it stems from a conference, in this case one held in August 1986. But this is not simply a book of conference proceedings. It is a comprehensive, carefully edited volume, and as well as the 23 chapters (by a total of 47 contributors) it includes an extensive, integrated bibliography, a glossary and an index.

Clark Chapman and Faith Vilas, two of the editors, have written the first two chapters, presenting an excellent overview of the planet and discussing future observations and missions. Until recently Mercury had not been considered as a target by the National Aeronautics and Space Administration, the European Space Agency and the Soviet Union, but things may be changing, as clever new ways have been found to use multiple gravity assists from Venus and Mercury itself to manoeuvre an orbiter or lander craft to Mercury. Scientifically exciting missions are therefore possible with existing launch vehicles (Titan/Centaur, Ariane V, or Proton); but it remains to be seen how the priorities for missions to Mercury will rank in comparison to those for Mars, Saturn or comets in a period of limited spending for space science and exploration.

Meanwhile this book provides an excellent summary of our current knowledge of the planet. Several contributors discuss its geology, comparing its history of impact cratering and volcanism with that of the Moon, which it superficially resembles. Mercury's unique compressional scarps tell a tale of internal shrinking due to cooling and phase changes, while the presence of a global magnetic field requires a partially liquid core and places constraints on bulk composition. Also discussed are the recently discovered atmosphere of sodium and potassium, and interactions between the magnetic field of the planet and the solar wind. Perhaps most interesting are the chapters that synthesize the data on composition and structure to develop theories of Mercury's formation and evolution, including the giant impact hypothesis for removal of much of its silicate shell. The treatment is authoritative and comprehensive, providing within one cover very nearly all we know — although certainly less than planetary scientists would like to know — about this remarkable planet. □

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Analysis under analysis

Michael B. Richman

Principal Component Analysis in Meteorology and Oceanography. By Rudolph W. Preisendorfer. Posthumously compiled and edited by Curtis D. Mobley. Elsevier: 1988. Pp.425. Dfl.210.

PRINCIPAL component analysis (PCA) is a powerful multivariate technique that can be applied to a range of problems in the social and physical sciences. It has roots in fields as diverse as statistics, physics, biology, psychology and meteorology, and has undergone parallel evolution in each of them.

Rudolph Preisendorfer's ability to assimilate and distil information from a wide array of subject areas and identify a common denominator is revealed in this book. It is a predominantly theoretical text, with enough applied material to help the reader grasp the importance of the derivational work. The first four chapters outline in detail the algebraic foundations of PCA, and its dynamical origins and multivariate formulations. One difficulty in compiling this type of book, dealing with interdisciplinary aspects of eigenvector research, is the varied and contradictory jargon used — one researcher's factors may be another's components and a third's empirical orthogonal functions (EOFs). Thankfully, at the outset Preisendorfer sets a convention for terminology, deciphering many articles so that their message is not lost.

As well as the derivation of PCA, Section 2 presents the origins of singular value decomposition and demonstrates its importance in bridging PCA and EOFs. Interspersed with the theory are several useful examples to help cement the concepts. Chapter 3 provides classic examples from Preisendorfer's earlier work on the dynamical origins of simple systems to illustrate properties of PCA, which are then generalized to vector fields in Chapter 4.

One of the highlights of the book, found in Chapter 5, is a critical examination of a range of PC truncation selection rules developed to separate signal from noise. There are several subsections which contain helpful comments, comparisons, and caveats for groups of rules. This chapter, combined with the bibliographical notes, will be required reading for anyone who is interested in gaining a full understanding of both the older and current methods of separating signal from noise in eigenanalyses.

The next few chapters examine connections between PCA and the related techniques of factor analysis (FA), canonical

correlation analysis (CCA) and linear regression analysis (LRA). Much of the material on FA is fashioned after Lawley and Maxwell's *Factor Analysis as a Statistical Method* (Butterworths; 2nd Edn 1971), and shares their embracement of the maximum likelihood method. The relevant bibliographical notes are well worth turning to for added insight, though readers wanting a full view of factor methods and problems associated with parameter estimation and indeterminacy will need also to refer to texts such as Mulaik's *The Foundations of Factor Analysis* (McGraw Hill, 1972).

Chapter 7 provides an introduction to linear transformations which can be applied to either PCA or FA. Preisendorfer cites work by Buell as the reason for going beyond unrotated PCA solutions with specific algorithms (Varimax and Procrustes rotations) being discussed in detail. Again, because this chapter is intended as an introduction to the topic of linear transformations, a few nuances are missing; for example there is no discussion of the role of hyperplanes (as contrasted to clusters) or that of pairwise graphical plots in explaining and validating simple structure. Yet the chapter does offer several glimpses of techniques that are only now beginning to be used by geophysical scientists, for example Procrustes Target Analysis and extended applications of Rule N with the Principle of Mimicry. The derivation of CCA in a PCA framework (Chapter 8) facilitates understanding of an aspect of CCA in applied research, and there is also a section on CCA selection rules which contains previously unpublished material. Chapter 9 similarly contains unique discussions of PCA regression, hindcast skill, and how this relates to CCA.

The last three chapters deal with statistical-dynamical models, partitioning eigenvectors and complex harmonic PCA. Chapter 10 is an impressive examination of two examples of substituting eigenmodes into models of atmospheric flow. Likewise, Chapter 12 contains a wealth of information on complex PCA, motivated by the thought that "we may reasonably expect that on combining the techniques of PCA and harmonic analysis, something of greater applicability than each will result for certain diagnostic or predictive tasks".

This is a well-organized and well-written text which draws information from half-a-dozen fields and brings it together into a concise statement on the theory and geophysical uses of principal components. We should be indebted to Curtis Mobley for bringing it to fruition after Preisendorfer's untimely death. □

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The ecological solution

Alex Kacelnik

Dynamic Modeling in Behavioral Ecology. By Marc Mangel and Colin W. Clark. Princeton University Press: 1989. Pp. 308. Hbk \$45, £28; pbk \$15.95, £10.

STOCHASTIC dynamic programming (SDP) is a technique to find optimal solutions when the payoff of alternative actions is probabilistic, conditional on the state of the optimizing agent and dependent on the time of the action. It has been used in behavioural ecology for more than a decade, and in other fields for much longer. So can there be much excitement about a new book on the subject? In this case, the answer is 'yes'. Mangel and Clark's volume combines the clarity of an extremely pedagogical — indeed at times even patronizing — textbook with insightful accounts of the application of SDP to a variety of biological problems.

The section on fundamentals, in spite of requiring little previous mathematical knowledge, succeeds well in describing the probability concepts needed to tackle a broad range of stochastic problems. The authors even explain how to write a computer program. This section will show those biologists who claim that they cannot get involved in formal modelling, because of a lack of expertise in mathematics, how wrong they are. It also includes a discussion of the classical optimal-foraging problem of patch selection, treated in the framework of SDP. Without explaining the principles, the authors lead the reader gently through implementation of a full SDP model. People familiar with state-independent, rate-maximizing solutions will be impressed by the understanding that this simple model provides.

The core of the book describes applications of SDP to various biological problems, including hunting in lions, reproductive strategies in insects, vertical migrations of plankton and planktivorous animals, parental care in birds, and dispersal in spiders and raptors. Each example is clearly presented and carefully chosen to introduce a new awareness of the resources available to the modeller. Although the intention is to provide an introduction to the models, not to justify each one in detail, and although much of the material has been presented elsewhere, this section includes many novel additions and in itself contributes substantially to the various areas of research involved.

Treatment of the principles and extensions of SDP follows the applications in the core of the book. This may seem an

unusual sequence, but it works well. Instead of being over-loaded with information before its possible uses are made clear, the reader is enabled to appreciate the potential applications of each method by generalization from particular cases.

As the authors explicitly point out, SDP is not the only way of tackling problems of optimality in behavioural ecology. In an effort to be fair, they include a brief discussion of other methods, but their sympathies are obvious and one by one other techniques are shown to be less promising than SDP. Newcomers to modelling, however, should be made aware that other approaches can, in some circumstances, be preferable. For example, solutions obtained by SDP usually (but not only) come as tables showing what the agent must do if a

number of conditions are met. These tables tend to be rather unwieldy when the main interest is the link between optimal behaviour and psychological or physiological mechanisms; although alternative approaches such as control systems theory are not as user-friendly when one is calculating optimal solutions, they do make it easier to cross levels of integration.

This is not a criticism of Mangel and Clark, who are mostly involved with optimality problems; rather it is a note of caution which I voice because of the very success that I expect and wish for this excellent book. It should have a considerable influence on the spread of theoretical modelling in behavioural ecology. □

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A light touch

S. D. Smith

Optical Computing: A Survey for Computer Scientists. By Dror G. Feitelson. MIT Press: 1988. Pp. 393. \$37.50, £29.95.

THIS is a remarkable book. Written by a computer scientist, it deals with a subject that is currently only a research topic and that spans several disciplines in optical physics and information technology. Essentially it is a survey of the field written by an outsider for outsiders; but such is its quality that in my opinion it should be standard reading for researchers themselves.

Feitelson begins with a short introduction, making a few general points about why optics might be interesting in the field of computing, and giving some guidance to relevant journals. He then continues with an unexceptional but well-written 50-page background account of optics.

A comprehensive chapter on optical image and signal processing follows which is well placed in the context of the subject. The author clearly realizes the importance of harnessing the two-dimensional capability of optics. He provides excellent descriptions of optical Fourier transform procedures and pattern recognition, including cross correlation, optical frequency plane correlators and matched filtering techniques. This topic is thoroughly treated, and the chapter extends to synthetic aperture radar imaging and radio signal analysis. Optical processing technology has, of course, had some success in practical systems for these applications, but we still await more powerful real-time technology.

The fourth chapter deals with numerical processing by optical methods, and is a useful listing. Feitelson briefly considers the combination of optical and electronic

systems by describing not only what optics can do for computing but also what electronics can do for optical processing. His remarks about the value of electronic control of optical logic planes are to the point, but he seems to have missed the concept of a 'lock and clock' architecture, which is necessary to protect information transmitted at the speed of light from being corrupted. The review of the interaction of nonlinear optics on possible future digital optical technology specifically excludes any discussion of the physical mechanisms of such nonlinearities. Because it is these mechanisms that control switching energies and operating powers, the author restricts himself to reporting the literature as read.

The state of research is faithfully represented, however, although I would like to have seen some question marks raised over the small amount of existing experimental work on digital optical circuits — that which has been done *has* shown that indefinitely extensible restoring optical logic is practicable. The general importance of the spatial light modulator and its digital equivalent, the optical logic plane, comes through well.

The book ends with a sensible discussion of the place and role of optical computing, some useful appendices and a large and up-to-date reference list. Altogether, Feitelson has written a most useful volume which deserves wide circulation. □

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Second editions

● *Earth and Life Through Time* by Steven M. Stanley. Publisher is W. H. Freeman, price is hbk \$39.95, £31.95, pbk \$26.95, £18.95. For review see *Nature* 319, 811 (1986).

● *An Introduction to Chaotic Dynamical Systems* by Robert L. Devaney. Publisher is Addison-Wesley, price is £34.95.

The Spitak (Armenia) earthquake of 7 December 1988: field observations, seismology and tectonics

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The epicentre of the destructive earthquake that devastated northern Armenia, the strongest in the region since historical times, is located within the Lesser Caucasus, a mountain country subjected to north-south compression by the push of the Arabian plate. A French-Soviet field expedition studied surface breaks and aftershock activity. The fault scarp could be followed for 13 kilometres and showed a reverse dislocation of 1.6 metres. Aftershocks are shallower than 13 kilometres, and delimit a ruptured surface of about 300 km².

A DESTRUCTIVE earthquake devastated the region around the cities of Spitak, Leninakan and Kirovakan in northern Soviet Armenia on December 7, 1988, at 07:41 UT (latitude 40.94° N, longitude 44.29° E, depth 10 km according to the US National Earthquake Information Center (NEIC); 41.15° N, 44.25° E after the Euro-Mediterranean Seismological Centre; 40.84° N, 44.32° E, depth 10 km using 19 local Soviet stations). Its magnitude was 7.0 according to the Seismological Centre of Obninsk¹, 6.9 according to NEIC and 6.7 according to the Institut de Physique du Globe de Strasbourg. This was the largest earthquake in this region since historical times. Official figures give 25,000 people dead, the city of Spitak being destroyed to 90%, Leninakan to 50% and Kirovakan to 20%. The maximum intensity, $I_0 = X$ in the MKS scale, was observed in the region to the north-west of Spitak.

A French-Soviet team formed by seismologists and geologists went to the source region 10 days after the main shock in order to register aftershock activity and to map the surface breaks (Soviet seismologists and geologists arrived at the epicentral zone on 10 December). A similar American group from the US Geological Survey² also worked in the area.

Geodynamics

The epicentral zone is located within the Lesser Caucasus, which is dominated by a north-south compressive tectonics resulting from the young continental collision between the Arabian plate and the Russian Platform³. The rate of convergence has been evaluated at 3 cm yr⁻¹. Figure 1 illustrates the main features of this collision; there is lateral ejection of the Turkish block to the west along the North Anatolian Fault, the Iranian block to the east, and the squeezing of a region about 800 km wide

between the northern front of the Arabian wedge and the Russian Platform.

The Lesser and Great Caucasus are located to the north of this region. Caucasian tectonics is characterized by reverse faulting and folding associated with large strike-slip faulting. Present and neogene tectonics of northern Armenia shows that north-south compression coexists with east-west extension related to recent volcanism along north-south-oriented normal faulting, and with the Borjomi-Kasbeg left-lateral strike-slip fault³.

The earthquake fault belongs to the approximately east-west Sevan-Akera deep thrust zone, the old Tethys suture, which borders the Lesser Caucasus to the south, running along the northern border of the Sevan Lake and to the north of the Aragat volcano and volcanic plateau. The Pambak river runs along the fault zone, the structural disposition of its basins suggesting a right-lateral component of the motion across the fault.

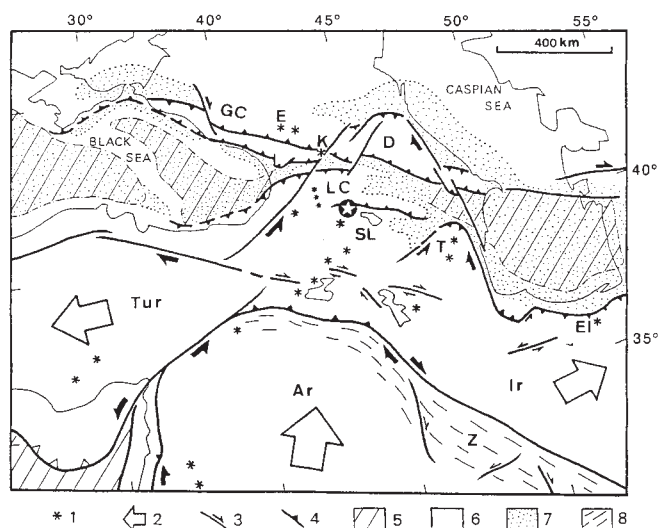


FIG. 1 Present-day tectonic features characterizing the Caucasus. The star shows the epicentre of the 7 December 1988 Armenian earthquake. 1, Recent volcanoes; the Aragat volcano is just below the epicentre. 2, Relative motion with respect to Eurasia. 3, Major strike-slip faults. The north-east-oriented Borjomi-Kasbeg fault passes next to the Kasbeg volcano. 4, Major thrusts; the Sevan-Akera fault zone passes by the epicentre of the Spitak earthquake. 5, Oceanic or intermediate crust. 6, Continental crust. 7, Main sedimentary basins. 8, Recent folding at the border of the Arabian plate. GC, Great Caucasus; D, Dagestan; LC, Lesser Caucasus; T, Talesh; EI, Elbrus; Ir, Iranian block; Tur, Turkish block; Ar, Arabian plate; Z, Zagros; SL, Sevan Lake; K, Kasbeg volcano; E, Elbruz volcano.

The main shock

The main shock was preceded by a foreshock of magnitude $M_L=3$, on 6 December at 15:27, and was followed 4 min 20 s later by a large aftershock of magnitude $M_L=5.8$ (ref. 1). Different focal mechanisms have been calculated by different groups, the first being obtained by the analysis of surface waves. Two days after the earthquake, a mechanism (Fig. 2a) was obtained⁴ using Love and Rayleigh waves at periods ranging from 20 to 50 s (ref. 5). This result (azimuth = 309°, dip = 29° for the fault and slip vector = 107°) was conveyed to NEIC in Golden, Colorado, and to the Institute of Physics of the Earth in Moscow. The thrust component was shown clearly on this first solution, although there was no resolution of the horizontal

component of the slip, and the half-depth of the source was found to be 7 km. A week later, the global Geoscope⁶ network permitted a more constrained mechanism⁷ to be obtained by using Rayleigh waves retrieved by dial-up teletransmission from eight stations at a longer period ($180 < T < 320$ s) and at a fixed depth of 10 km. Figure 2b shows this solution, which is remarkably close to what was observed in the field (azimuth = 299°, dip = 72° for the fault and slip vector = 135°). The residuals for the amplitude at different azimuths were small, and the phase was well explained except at those azimuths where rapid variations occur. The seismic moment was 1.6×10^{26} dyne cm for the single-station Strasbourg solution, and 2×10^{26} dyne cm for the Geoscope determination. With these values, the expected length of the fault should be of the order of 20 km if we estimate a mean slip of 2 m and a fault width of 15 km from field observations. Broad-band body-wave modelling, obtained four months later when the records became available, is shown in Fig. 2c (azimuth = 30°, dip = 60° for the fault plane and slip vector = 110°). The P and SH waves from broad-band and long-period stations, low-pass-filtered at 6 s, are well explained by two pulses, each with a duration of 3 s and a seismic moment of $\sim 0.4 \times 10^{26}$ dyne cm, separated in time by 12–16 s. A small precursor is needed to fit the beginning of the signal. Separation between the pulses is larger in the Chinese stations than in the African, European and American ones, suggesting that the rupture propagated from east to west.

Besides the tectonic evidence, we observed that focal mechanisms in the Caucasian region, those calculated by Jackson and McKenzie⁸ for example, are compatible with a north-south compression. The mechanism of the Spitak earthquake thus gives new, high-quality additional information to confirm the general features of the tectonics of the region.

Surface breaks

Surface breaks oriented roughly 120° N have been observed between the town of Spitak and the village of Gekhasar (Fig. 3). They show a reverse faulting dipping to the north with a right-lateral offset. The overall situation is rather simple in comparison with other well documented reverse-faulting earthquakes, such as El Asnam⁹ or Tabas¹⁰.

The surface breaks present a narrow band of pressure ridges within the soil cover and alluvians (Fig. 4a), whereas a neat scarp is observed within the bedrock (Fig. 4b). The fault plane dips to the north at an angle of between 50° and 80°. Striations on fault faces are consistent with reverse motion with a slight right-lateral component when the azimuth is 120° N, and with pure reverse motion when the strike is east-west. Vertical and horizontal displacements increase from north-west to south-east. We observed 40 cm of vertical displacement and 15 cm of right-lateral offset near Gekhasar, but 160 cm of vertical displacement and 40 cm of right-lateral displacement near Spitak. Shortening of about 70 cm was measured across an irrigation duct at about 1 km to the east of Gekhasar. Some secondary features near Gekhasar consist of normal faulting within the thrusting block resulting from the collapse of the hanging compartment.

We have mentioned that the seismic-moment calculation pointed to a rupture surface of ~ 300 km². Then, if the aftershocks are shallower than 15 km, the fault length should be at least 20 km, which is greater than what is observed at the surface. This discrepancy must be explained. No continuation of the fault breaks have been found east of Spitak up to now. On the other hand, a 200-m break effects an anticline hinge about 5 km west-northwest of Gekhasar (Fig. 3). It is possible that the broken area appears only partially at the surface, but also that brittle rupture is replaced by continuous plastic deformation. If the fold is the surface expression of the blind thrust existing at depth, then the total length of the thrust becomes 16 km, in better agreement with the estimations of seismic moment.

The observed fault separates different geological formations at most places (Palaeocene volcanic rocks in the northern block,

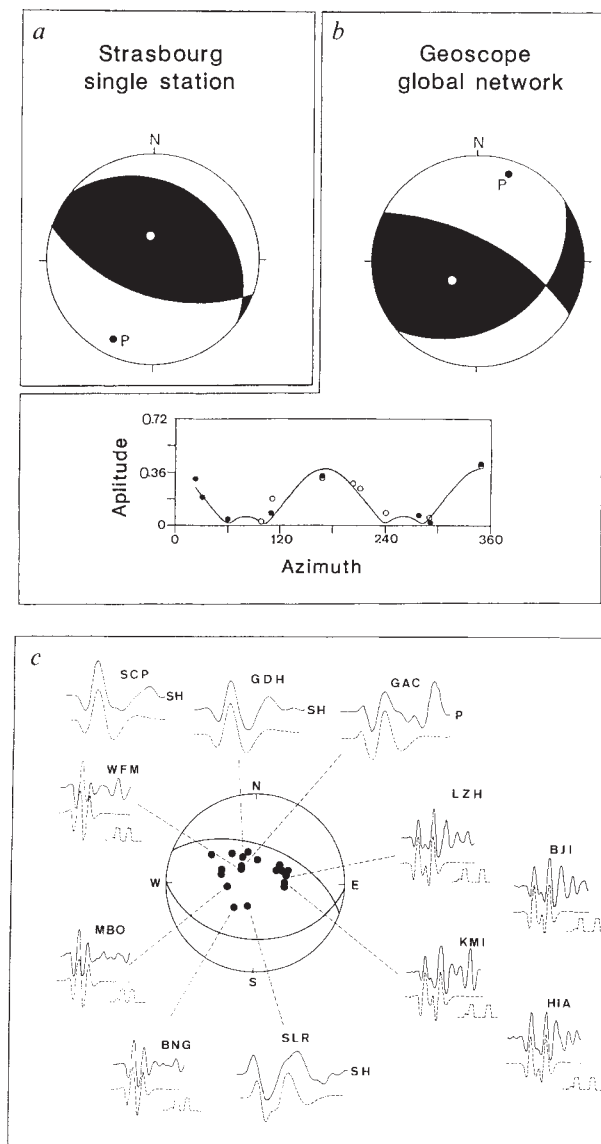
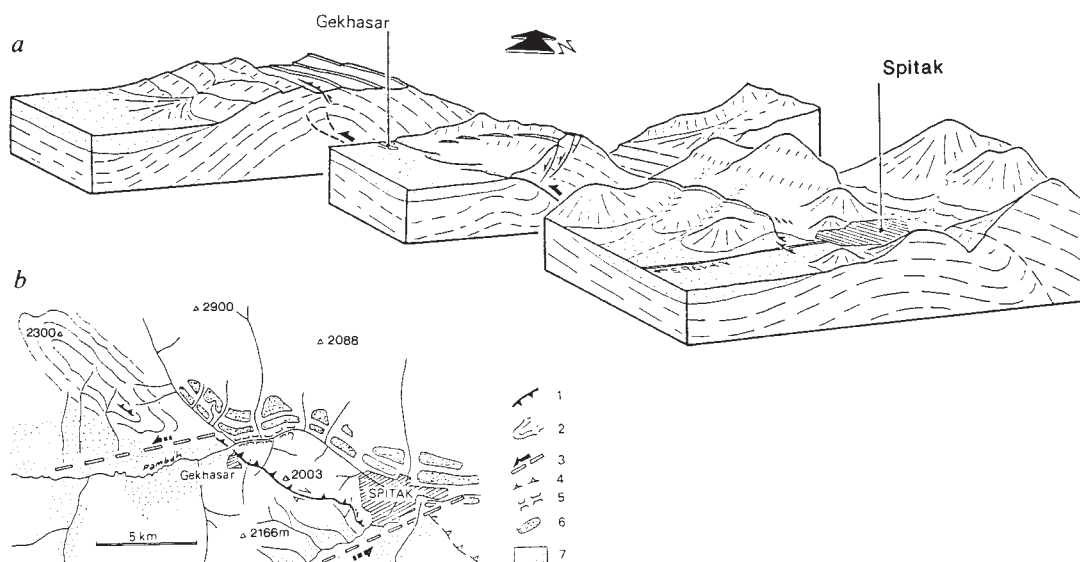


FIG. 2 Focal-mechanism solutions for the main shock. The circles represent the horizontal projection of the lower hemisphere of a sphere centred at the source. The fault plane and a plane orthogonal to the slip are shown. Dark areas are regions in which the source pushes towards the station. a, Strasbourg solution from analysis of surface waves of the Echery broad-band records 48 h after the earthquake. b, Solution obtained one week later from Rayleigh-wave data from 8 globally distributed Geoscope long-period stations. Lower figure shows the fit of amplitude as a function of the azimuth at the source. c, Broad-band body waves and long-period P and SH waves from the GDSN and Geoscope network. Solid lines are recorded seismograms, and segmented lines are theoretical. The model consists of two pulses, 3 s wide, separated by 12 (MBO and BNG) to 16 s (Chinese stations LZH, BJI, KMI and HIA), preceded by a small precursor.

FIG. 3 Surface breaks and deformation related to the main shock. *a*, Block diagram showing surface ruptures between Spitak and Gekhasar and the fold to the west of Gekhasar. A geological fault exists to the east of Spitak but was not activated during the earthquake. *b*, Same as *a* but on the horizontal plane. High points are indicated, along with their elevation in metres. 1, Thrusts activated during the earthquake. 2, Layering within the anticline west-north-west of Gekhasar. 3, Inferred left-lateral strike-slip faults offsetting the segments of the thrust. 4, Thrusts not activated during the main shock. 5, Pambak river gorge. 6, Uplifted Quaternary terraces. 7, Quaternary sedimentary filling in subsiding valleys.



and upper Cretaceous limestones to the south). Therefore it is likely that the fault acted as a normal fault in the past, and that the motion later changed to reverse. The limestones are distorted and folded against the fault gauge at the contact, in agreement with the recent sense of motion (Fig. 3). There is no geomorphological evidence for a fault scarp along this geological contact. Nevertheless this fault has been an active thrust during Quaternary times, as indicated by the exposures along the road from Spitak to Leninakan, near Gekhasar. In fact, Quaternary volcanic tuffs and alluvial deposits are faulted and tilted near the fault trace. A further and more striking piece of evidence is the overall disposition of fluvial deposits on both sides of the fault. To the north of the fault trace, or its continuation, there is a system of well developed successive terraces that have been elevated and then eroded by the Pambak river and tributaries. On the other hand, south of the fault trace, the valleys are smoothly filled with sediments, swamps are present and no terraces are observed. This disposition indicates that during Quaternary times the southern compartment of the fault subsided but the northern one was uplifted. As this earthquake is the largest observed during historical times, it is certain that no conclusions regarding seismic hazard may be obtained from the historical record alone. It is then necessary to appeal to

palaeoseismic studies to estimate maximum possible earthquake and recurrence times.

Aftershocks

A seismic network of 25 portable stations and 3 accelerometers was arrayed around the source region by the French team 10 days after the main shock (Fig. 5). The network, with a diameter of about 50 km, consisted of 10 MEQ analog recorders with L4C Mark Product seismometers, six Geostars digital three-component autonomous stations recording on magnetic tape, and a portable digital telemetric network, built in Strasbourg, with one three-component and eight vertical-component stations centred at Spitak.

Day-to-day location of the aftershocks was routinely performed during the field experiment together with magnitude determination and observation of other parameters of the seismic regime. In Fig. 5 we show the distribution of 259 well recorded aftershocks corresponding to the period from 22 December 1988 to 1 January 1989. The relative disposition of the events with respect to the surface deformations and breaks indicates that most of the activity is concentrated to the north of the surface scarps. The aftershock cloud is elongated along the trend of the fault. One notable feature is the very low level of activity



FIG. 4 Representative surface breaks looking towards the west. *a*, Pressure ridges on soil. Extension cracks indicate a right-lateral component of slip. *b*, Scarp in volcanic rock showing 1.6 m of reverse vertical offset. Bushes

were burnt next to the fault trace, possibly because of escape and ignition of gases through tension cracks.

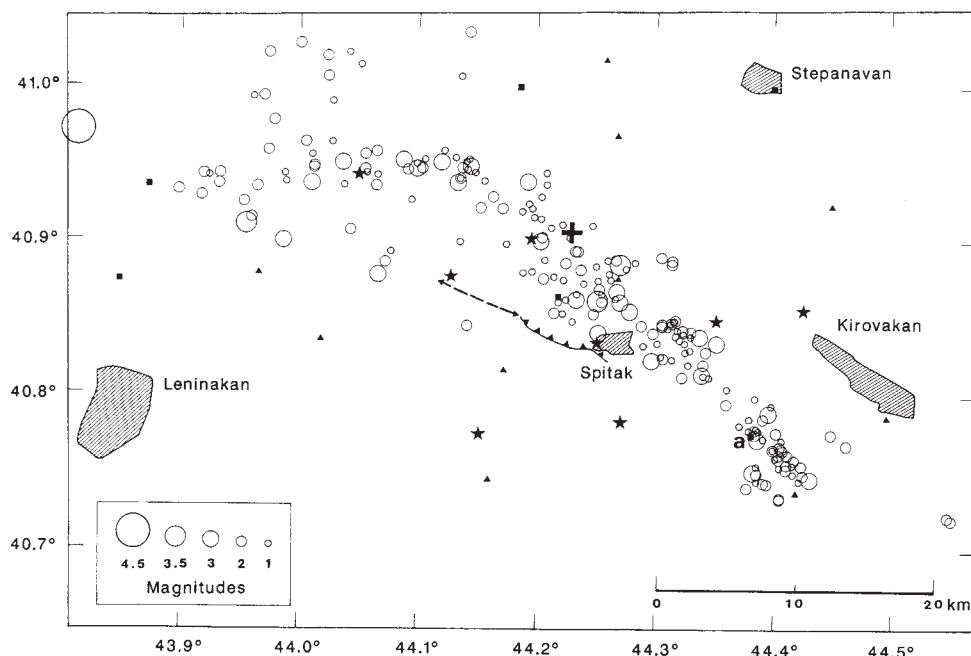


FIG. 5 Space distribution of aftershocks within the period from 22 December 1988 to 1 January 1989. The seismic network is composed of eight telemetric digital stations centred at Spitak (stars), six Geostars three-component digital stations (squares) and ten analog Sprengnether MEQ stations (triangles). The epicentre of the main shock, localized relative to the 31 December aftershock, is shown as a black cross. The fault trace, the fold axis (dashed line) and the cities of Spitak, Leninakan, Kirovakan and Stepanavan are shown for reference. **a**, denotes the Aidarli station.

in the vicinity of the rupture zone or its estimated extension to the west into the fold. Aftershocks concentrate on the border of this gap. It is reasonable to assume that this surface corresponds to the zone of rupture during the main shock. The area of surface involved is $\sim 300 \text{ km}^2$, in agreement with the estimations from the seismic moment. Further clustering is observed in the neighbourhood of Spitak and also to the east near Aidarli. These clusters are separated by a gap that may correspond to one of the strong aftershocks. Most of the aftershocks are shallower than 13 km (Fig. 6a). A cross-section orthogonal to the fault trace shows that there is a concentration of aftershocks near a plane dipping 50° to the north (Fig. 6b).

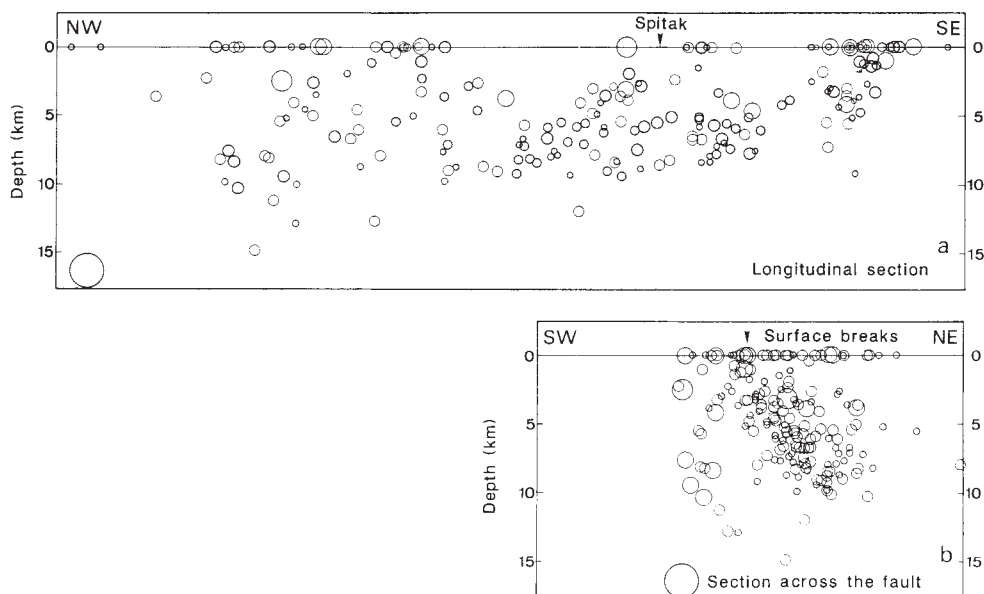
Discussion

The fault of Spitak is simpler than that of Al Asnam⁹ and has produced few secondary features. Nevertheless, it is segmented and we find that at least two segments moved during the main shock. One corresponds to the fault breaks between Spitak and Gekhasar, and the other to the blind thrust to the west of Gekhasar. The almost total destruction of Spitak is certainly related to the fact that the trace of the fault passed along the

border of the town. A third segment, to the east of Spitak, is clearly recognized on the field as the continuation of the Spitak fault, but showed no evidence of surface rupture in spite of the cluster of aftershock activity that may be associated with it. It is likely that these segments are offset and separated by left-lateral strike-slip faults (one at least being evident on a SPOT satellite image) trending 80° N and forming a tectonic system that is kinematically consistent with a north-south compression (Fig. 3b). This scheme is substantiated by the drainage network, which follows the fault system. The sense of propagation of the rupture will be discussed in detail elsewhere; as the main shock has a relative location close to the end of the fault, near Spitak (Fig. 5), we conclude that the rupture propagated from east to west. The largest aftershock, 4 min 20 s after the main shock, is located to the east of Spitak, and may thus explain the gap and clusters near Aidarli.

The occurrence of the Spitak earthquake draws attention to the problem of determining the maximum possible earthquake in the region. This is the strongest earthquake in northern Armenia since historical times¹¹. Spitak was affected by an earthquake of magnitude 5 in 1967, Kirovakan suffered a shock

FIG. 6 Vertical distribution of aftershock activity. **a**, Section along the fault trace. Hypocentres are not deeper than 13 km. **b**, Section across the fault scarp. The geometry suggests a fault plane dipping about 50° to the north. Note that some shocks have an automatically fixed zero depth.



of magnitude 5.3 in 1911 and Leninakan was the site of an earthquake of magnitude 5.7 in 1926. Few seismologists believed that an earthquake as severe as that of 7 December 1988 was possible in this region. It is clear that seismic history alone is not sufficient to arrive at such conclusions. One way to improve the assessment of seismic hazard in this region, where continental collision is dominant and seismicity is confined to the crust, is to increase the knowledge of active faulting, for example by improvement of the permanent seismic network, neotectonic mapping of Quaternary deformation, analysis of aerial and

satellite images, and the use of palaeoseismology in appropriate sites to give an insight into past activity.

Pattern-recognition analysis of earthquake-prone areas made by the Soviet group for events of magnitude $M > 6.5$ in Armenia classified as dangerous an object around Leninakan that included the epicentre of the 7 December Spitak earthquake¹². Further study¹³ established an object close to Erevan as being dangerous for earthquakes of magnitude $M > 7$. It is now recommended that this latter region also be subjected to short-term prediction studies, because a nuclear power plant is operational there. □

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Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2

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Actively transcribing eukaryotic RNA polymerase II is highly phosphorylated on its repetitive carboxyl-terminal domain. We have isolated a protein kinase that phosphorylates serine residues in this repetitive domain. A component of this kinase is cdc2, the product of a cell-cycle control gene previously shown to be a component of M-phase-promoting factor and M-phase-specific histone H1 kinase. This observation suggests a role for the cdc2 protein kinase in transcriptional regulation.

THE largest subunit of eukaryotic RNA polymerase II (RNAP II) contains an unusual carboxyl-terminal domain (CTD) comprising multiple repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This domain is not found in other classes of eukaryotic RNA polymerases or in prokaryotic RNA polymerases. The heptapeptide sequence is repeated, with some degeneracy, 52 times in mouse¹ and Chinese hamster², 42–44 times in *Drosophila*^{2,3} and 26–27 times in yeast^{4,5}. Deletion mutations that result in the loss of more than half of the repeats in mouse and yeast are lethal, indicating that this domain plays an essential role in RNAP II function *in vivo*^{2,5,6}. Although the nature of this essential function is unknown, actively transcribing RNAP II is known to be highly phosphorylated on the CTD^{7,8}.

Three forms of RNAP II (IIo, IIA and IIB) have been described each differing in the apparent relative molecular mass

(M_r) of the largest subunit: IIo, 240,000 (240K); IIA, 215K and IIB, 180K. Several lines of evidence have shown that the subunit IIB is derived from the subunit IIA through proteolysis of the CTD^{1,9,10}. RNAP II purified by standard techniques consists mainly of forms IIA and IIB; however, if immunoblots of proteins from lysed nuclei are probed with antibodies against the largest subunit, the predominant form is IIo (refs 11, 12). Furthermore, photoaffinity-labelling of actively transcribing RNAP II in *in vitro* extracts or in nuclei reveals primarily subunit IIo, indicating that IIo is the most transcriptionally active form of the enzyme *in vitro* and *in vivo*^{7,8}.

In vivo labelling studies have shown that the IIo form of the RNAP II largest subunit is highly phosphorylated¹³. Subunit IIo can be converted to IIA by phosphatase treatment, indicating that the higher M_r of IIo is a result of phosphorylation⁸. Analysis of a cyanogen bromide fragment containing the CTD has shown that this domain is multiply phosphorylated on both serine and threonine residues⁸. Thus, IIo appears to be derived from IIA by phosphorylation of the CTD. To understand the role of CTD phosphorylation in RNAP II transcription, we initiated a search for protein kinases that phosphorylate this domain. We report here the isolation of a CTD kinase from mouse ascites tumour cells and demonstrate that the mouse cdc2 protein is a component of this enzyme.

Development of a CTD kinase assay

The CTD is comprised almost entirely of tandem heptapeptide repeats with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Reasoning that one or a few of the heptapeptide repeats would, in isolation, adopt a structure similar to heptapeptides within the CTD, we decided to use chemically synthesized heptapeptides as potential protein kinase substrates. The

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sequences of peptide substrates used in this paper are shown in Fig. 1a. Synthetic heptapeptides hepta-4 and hepta-6 were used as substrates in phosphate transfer reactions in the presence of [γ - 32 P]ATP and various cell extracts. Labelled proteins from these reactions were separated on SDS-polyacrylamide gels and autoradiographed. Figure 1b shows the results of reactions containing hepta-4, hepta-6 or no added peptide. In the two lanes containing peptide a new labelled band is apparent. The mobility of the peptide-dependent band is proportional to peptide length, strongly suggesting that the labelled band is phosphorylated peptide. This result demonstrates that cell extracts contain an activity that phosphorylates the heptapeptide repeat. This kinase activity is magnesium dependent, cAMP and calcium independent, and displays broad pH and salt optima.

Figure 1b also demonstrates the aberrant electrophoretic mobility of the labelled peptides. On a 12.5% (30:1 acryl-

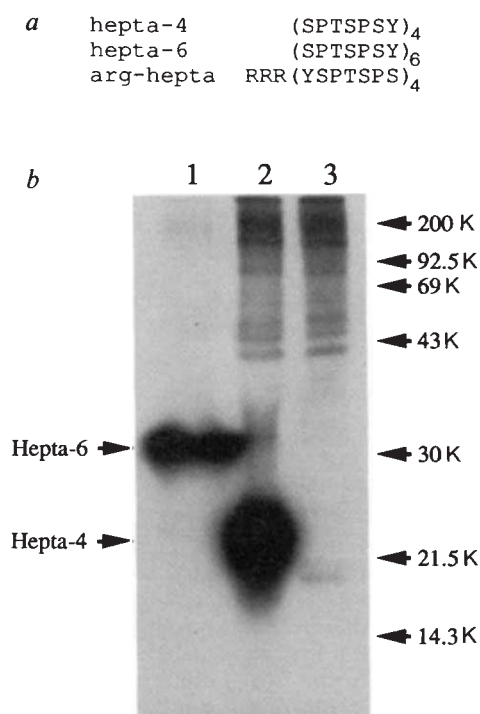


FIG. 1 Phosphorylation of heptapeptide substrates. *a*, Sequences of heptapeptide substrates. Peptides were prepared on an Applied Biosystems model 430A peptide synthesizer using t-Boc chemistry. Following synthesis, peptides were cleaved from the solid support, deblocked using hydrogen fluoride, and chromatographed on a Vydac reverse-phase preparative C₁₈ column with a gradient of 0.1% trifluoroacetic acid (TFA) in water to acetonitrile/2-propanol (2:1)/0.1% TFA. Appropriate fractions were pooled and, for peptides hepta-4, and hepta-6, and subjected to a second round of reverse-phase chromatography using a Vydac analytical C4 column using the same solvent system. Arg-hepta was chromatographed on a Mono S (cationic exchange) column with a gradient of ammonium acetate as the second purification step. Peptides were then lyophilized, resuspended in water, and their concentrations determined from absorbance at 274 nm; the sequences were verified by automated Edman degradation using an Applied Biosystems model 470A protein sequencer and model 120 PTH analyser. *b*, Autoradiograph of SDS-PAGE of a whole-cell extract incubated with peptides hepta-6 (lane 1), hepta-4 (lane 2), or without added peptide in the presence of [γ - 32 P]ATP. The reactions contained 5 μ l mouse Erlich ascites cell whole-cell extract³⁹, 0.5 mg ml⁻¹ peptide, 60 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 0.1 mM dithiothreitol (DTT), 10 μ M [γ - 32 P]ATP at a specific activity of 25 Ci mmol⁻¹ in a final volume of 50 μ l. Reactions were incubated at 30 °C for 30 min, stopped by addition of SDS sample buffer containing 20 mM EDTA and boiling for 3 min. Samples were run on a 12.5% polyacrylamide gel⁴⁰ to resolve labelled proteins and peptides. The gel was fixed in trichloroacetic acid (TCA)/acetic acid/methanol/H₂O (10:10:30:50), dried on Whatman DE81 paper, and autoradiographed. Molecular weights are indicated on the right.

amide:bis) polyacrylamide gel, hepta-6, with a M_r of 4,314 (4,392 for monophosphorylated peptide), migrates adjacent to carbonic anhydrase, a protein of M_r 30K; moreover, hepta-4, with M_r of 2,876 (2,954 for monophosphorylated peptide), appears at a position equivalent to a protein of $\sim$ 23K. The M_r of the labelled peptides appears to be dependent on the acrylamide gel concentration (for example, see Fig. 3). Mobility of the labelled peptides is not dependent on the presence of SDS in the electrophoretic system. Thus, phosphopeptides apparently fail to bind SDS and therefore migrate on the basis of the charge (phosphate) to mass ratio. We presume that the peptide substrates shown in Fig. 1 are phosphorylated at a single site; longer incubation results in phosphorylation at multiple positions (up to once per heptapeptide repeat), which yields a series of bands with increasing mobility (data not shown). Sieving for these peptides is minimal at acrylamide concentrations of less than 12%. The aberrant mobility seen with these peptide analogues of the CTD may explain the high apparent M_r (240K) of the IIo subunit of RNA polymerase II.

For purification of the CTD kinase, we developed a quantitative and rapid filter-binding assay using the Arg-hepta peptide (Fig. 1a). In this assay, the labelled peptide substrate is removed from unincorporated ATP by binding the arginine residues of the peptide to phosphocellulose paper (Whatman P81). Unbound ATP is removed by washing and the filters are dried and counted¹⁴. This assay is equivalent to the electrophoretic analysis of labelled heptapeptide substrates because it detects the same peak of kinase activity in fractionated extracts, and, despite the presence of the three arginine residues, does not detect any new kinase activities. Moreover, as shown below, the purified kinase phosphorylates hepta-6.

Purification of a CTD kinase

To proceed with a large-scale enzyme purification, we screened a number of cell types and tissues to find a convenient starting source. We identified CTD peptide kinase activity in mouse, human, hamster and yeast cells but not in *Escherichia coli* extracts. The activity did not appear to be localized to cytoplasmic or nuclear fractions; therefore, a whole-cell extract was used as the starting material for purification. A whole-cell extract was prepared from mouse Erlich ascites cells and, following ammonium sulphate fractionation, the extract was subjected to DEAE-Sepharose chromatography. Because earlier reports indicated that casein kinases I and II are capable of phosphorylating the CTD *in vitro*¹³, we initially assayed column fractions for both casein kinase and CTD kinase activities. We observed two peaks of casein kinase activity on DEAE-Sepharose columns (data not shown). The first casein kinase activity did not bind to the column in the presence of 75 mM KCl, whereas the second casein kinase activity eluted at $\sim$ 300 mM KCl. CTD kinase bound to the column, unlike the first casein kinase, and eluted well before the second casein kinase. Thus, this column separated CTD kinase from the major casein kinase activities, presumably casein kinases I and II. Although these casein kinases have been reported to phosphorylate RNAP II *in vitro*¹³, we find very little heptapeptide kinase activity co-eluting with casein kinase. DEAE-Sepharose column fractions containing CTD kinase were pooled and successively chromatographed on heparin-Sepharose, Mono Q, phenyl-Superose, and Matrex Green A. Details of this purification process will be published elsewhere (manuscripts in preparation).

Figure 2 shows the results of the final column chromatography step. SDS-gel electrophoresis reveals prominent polypeptides of 58K and 34K in fractions containing CTD kinase activity. The main peak of CTD kinase activity (fractions 27–29) eluted from the affinity resin at 0.45 M NH₄Cl and coincides with the peaks of p58 and p34. On some gels we detect an additional polypeptide with M_r < 14K in these same fractions. The 65K and 90K bands seen in some active fractions clearly elute from the column before the CTD kinase activity, whereas the band

at ~85K, present in all fractions, is a gel artefact. An additional faint band of >200K is seen to peak with the kinase activity on this gel.

Several additional pieces of experimental evidence support the assignment of CTD kinase activity to a complex containing the 58 and 34K polypeptides. First, these polypeptides are not separated by gel filtration in buffers containing 2M urea or 900 mM KCl. Second, the CTD kinase has a native M_r of 80–100K as determined by gel filtration and isokinetic sucrose gradient analysis (data not shown). Furthermore, on non-denaturing sucrose gradients the 58K and 34K polypeptides co-sediment with the peak of activity, whereas the >200K and 90K polypeptides sediment ahead of the peak of activity. The amount of these larger proteins is also variable from preparation to preparation in a manner not related to CTD kinase activity. Together, these results suggest that the CTD kinase is a complex

containing 58K and 34K subunits and perhaps an additional smaller (<14K) subunit.

Specificity of CTD kinase

The ability of CTD kinase to phosphorylate RNAP II *in vitro* is demonstrated in Fig. 3a. Partially purified RNAP II was used as the substrate in a CTD kinase reaction. The largest subunit in this particular RNA polymerase preparation was partially dephosphorylated during purification resulting in a continuum of bands migrating between IIo and IIa (data not shown). When this RNAP II was incubated with CTD kinase in the presence of [γ - 32 P]ATP, a broad labelled band appears (lane 1) at a position between polymerase subunits IIo and IIa. This band is not present in either the polymerase alone (lane 2) or kinase plus peptide (lane 4) lanes. Furthermore, when kinase alone is incubated with [γ - 32 P]ATP a band of this size is not observed (data not shown). We therefore conclude that this band represents phosphorylation of the largest subunit of RNAP II by CTD kinase. This signal can be diminished by the addition of peptide (data not shown), making it likely that the CTD kinase recognizes similar sites on the heptapeptide and RNA polymerase substrates. Furthermore, we have recently shown that CTD kinase will shift the electrophoretic mobility of a recombinant CTD substrate from the position of the IIa CTD to the position of the IIo CTD (J. Zhang and J. Corden, unpublished results), indicating that CTD kinase could be the enzyme responsible for producing subunit IIo *in vivo*.

The nucleotide donor and amino-acid acceptor specificities of the CTD kinase have also been investigated. The enzyme will catalyse phosphate transfer from either [γ - 32 P]ATP or [γ - 32 P]GTP. We have estimated the K_m at 30 μ M and 180 μ M for ATP and GTP, respectively. Phosphoamino-acid analysis of purified labelled hepta-6 reveals serine to be the sole amino-acid acceptor (Fig. 3b). The K_m for hepta-6 has been estimated at 200 μ M.

To assess the relative specificity of CTD kinase, we have tested a number of other potential substrates for this enzyme. We have also tested heptapeptides as potential substrates for other protein kinases. Figure 3c shows the results of reactions in which heptapeptide, casein and histones were tested as substrates for purified CTD kinase, casein kinase II, and the catalytic subunit of cAMP kinase. A 10-fold mass excess of casein and histone (relative to peptide) was chosen to compensate for the higher serine content of the heptapeptide. Lanes 1–3 show that CTD kinase uses heptapeptide more efficiently than histone or dephosphorylated casein. Upon longer exposure we observe a weak signal in the histone lane (lane 2), and on higher-resolution gels this label is seen to migrate with both the core histones and histone H1 (data not shown). Casein kinase II (lanes 4–6) phosphorylates casein but fails to phosphorylate heptapeptide or histone, whereas cAMP kinase catalytic subunit (lanes 7–9) phosphorylates histone, and to a lesser extent casein, but does not phosphorylate heptapeptide to any detectable degree. We have tested CTD kinase for its ability to phosphorylate a number of other peptides and proteins, including degenerate heptapeptides (Tyr-Ser-Pro-Thr-Ser-Pro-X-Tyr-Ser-Pro-Thr-Ser-Pro-Ser, where X is Asn, Lys or Arg), angiotensin I, bradykinin, phosphovitin, protamine, polyserine, poly-Lys-Ser, poly-Arg-Tyr, poly-Orn-Tyr, and pol-Tyr. Only the degenerate heptapeptides, phosphovitin and protamine were found to serve as substrates for CTD kinase. These results indicate that the CTD kinase shows specificity for heptapeptide substrates and that these substrates are not universal protein kinase substrates. Further quantitative experiments will be necessary to rigorously define the CTD kinase substrate specificity.

Cdc2 is a component of CTD kinase

Based on the physical properties and substrate specificity of the CTD kinase activity, we concluded that this activity did not correspond to a known protein kinase. We therefore decided to

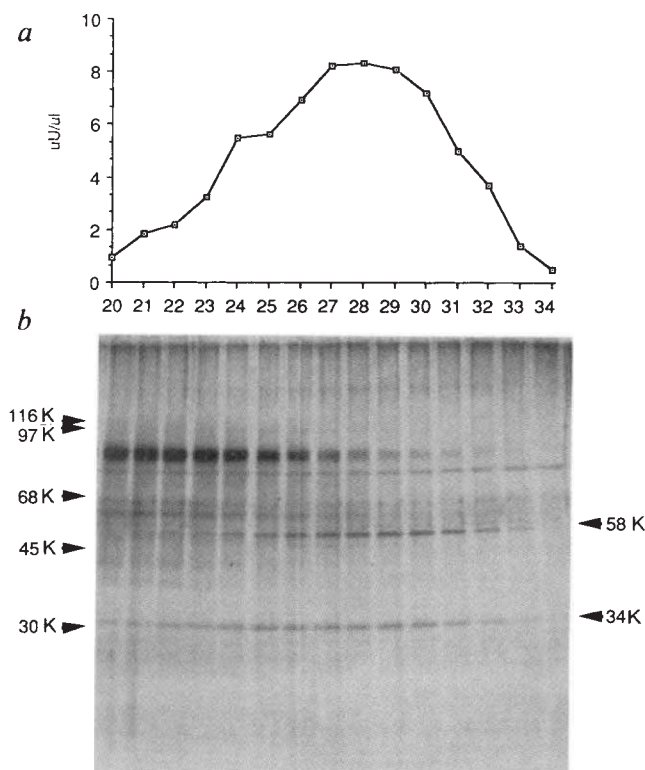


FIG. 2 SDS-PAGE of the final chromatography step in CTD kinase purification. *a*, Activity profile of CTD kinase from Matrex Green A column fractions. Active fractions from phenyl-Superose chromatography were dialysed against 100 mM NH_4Cl in buffer H (25 mM HEPES pH 7.4, 1 mM β -mercaptoethanol, 8 mM MgCl_2 , 10% glycerol). The dialysate was then applied to a 0.4 ml Matrex Green A column, washed, and eluted with a gradient of 100–1,000 mM NH_4Cl in buffer H. Diluted aliquots were assayed using Arg-hepta substrate. Assay conditions were 60 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM MgCl_2 , 0.1 mM DTT, 300 μ M Arg-Hepta, 200 μ M [γ - 32 P]ATP at a specific activity of 100 mCi mmol^{-1} . Reactions were incubated for 30 min at 30 $^\circ\text{C}$, stopped by the addition of an equal volume of 1M acetic acid saturated with EDTA, and spotted onto 2 $\times$ 2-cm squares of Whatman P81 paper¹⁴. The filters were washed five times in 75 mM phosphoric acid for 3 min each then rinsed with ethanol/75 mM phosphoric acid (1:1), and dried under a heat lamp. The filters were counted in the presence of scintillation cocktail. A unit of activity is defined as the quantity of enzyme needed to catalyse the transfer of 1 μ mol $\text{PO}_4 \text{ min}^{-1}$ under these conditions. *b*, Samples of the same fractions shown in *a* were reduced and denatured by boiling in the presence of SDS sample buffer, then run on a 9–17% polyacrylamide gel⁴⁰. The gel was silver-stained using the method of Morrissey⁴¹. The positions of M_r markers are indicated at the left, whereas the positions of the prominent 58K and 34K proteins are indicated at the right. A band occasionally observed at <14K was not visualized on this gel.

clone complementary DNAs encoding the polypeptides present in our most purified CTD kinase preparations. Our initial attempts focused on p34 because it was well resolved on preparative SDS gels at an early stage of purification. We performed *in situ* tryptic digestion on ~350 pmol of p34, and a number of tryptic peptides were purified by reverse-phase chromatography and sequenced¹⁵. The sequences for three peptides (TP14, TP16 and TP21) are shown in Fig. 4. These peptides all show striking homology to the predicted protein sequence of human cdc2 (ref. 16), a protein kinase involved in cell-cycle regulation.

A mouse *cdc2* cDNA clone was isolated by probing a mouse 3T3 cell cDNA library with a 51-base oligonucleotide containing the sequence of human *cdc2* spanning TP14 and the six amino acids immediately N-terminal to this peptide. One strongly positive clone was isolated and sequenced; the predicted protein sequence of this clone is shown in Fig. 4 along with the sequences of human *cdc2* (ref. 16), *S. pombe* *cdc2* (ref. 17), *S. cerevisiae* CDC28 (ref. 18), a partial sequence of a related human kinase (PSK-J3)¹⁹, and three of our tryptic peptides derived from p34. It is clear from the shaded sequence differences that the human and mouse sequences are the most closely related, having over 95% identical residues with no insertions or deletions in 298 amino acids.

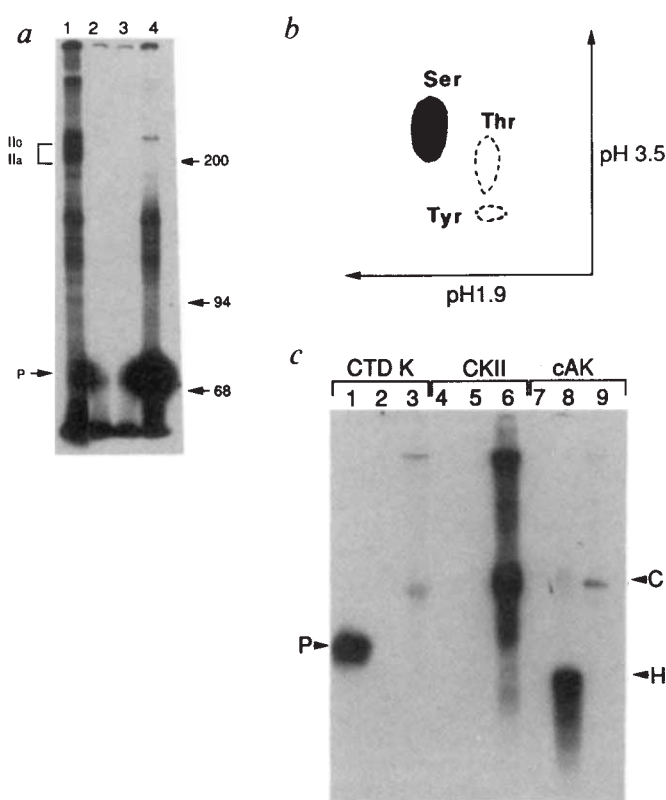
The amino-acid sequences of our p34 tryptic peptides support the conclusion that p34 is the product of the cloned mouse *cdc2* gene. The unambiguous portions of sequence for TP21 and TP14 are identical to the sequence predicted from the mouse cDNA clone, whereas that of TP16 differs at only one position. This single difference, an aspartic acid for glutamine at position 18 in TP16, may represent an error in peptide sequencing or,

alternatively, may be a genetic difference between the cells used for protein purification and library construction. The missing threonine residue in TP14 is probably the result of phosphorylation of this residue²⁰, ambiguous residues in TP16 are due to the low yield at later cycles, and the missing residue in TP21 was due to instrument error (histidine lag was detected in the subsequent cycle). In total, 36 residues were read from these three peptides and 35 of these amino acids are identical to the protein predicted from the cDNA sequence, strongly suggesting that p34 is the mouse *cdc2* protein. Other partially characterized peptides are also consistent with this assertion (data not shown).

To confirm that *cdc2* is a component of the CTD kinase activity, we used an antibody raised against the C-terminal seven amino acids of human *cdc2* (ref. 21) to immunoprecipitate the CTD kinase activity. Table 1 shows that this antiserum precipitates CTD kinase activity from a purified enzyme preparation and from crude extracts. The lower efficiency of precipitation of purified enzyme is either due to saturation of the antiserum with antigen or to partial proteolysis of the C-terminal residues of p34. The ability of anti-*cdc2* antibodies to immunoprecipitate CTD kinase activity strongly supports the conclusion that mouse *cdc2* is a component of a protein kinase that phosphorylates the CTD of RNA polymerase II.

We have not addressed the question of whether *cdc2* is the catalytic subunit of the CTD kinase complex. Although all of the conserved sequences common to protein kinases are contained within its coding region, the isolated *cdc2* polypeptide has not directly been shown to contain protein kinase activity; association with other subunits may be required for enzymatic activity.

FIG. 3 Specificity of CTD kinase. *a*, *In vitro* phosphorylation of RNAP II with CTD kinase. The reactions contained: lane 1, RNA polymerase II and CTD kinase; lane 2, RNA polymerase II; lane 3, RNA polymerase II and hepta-6; lane 4) CTD kinase and hepta-6. The positions of marker proteins are shown to the right, whereas the positions of the RNA polymerase II largest subunits (Ilo and Ila) and phosphorylated hepta-6 (P) are shown to the left. RNA polymerase II was partially purified by a modification of the method of Hodo and Blatt⁴² using polyethylenimine precipitation, DEAE chromatography, phosphocellulose chromatography, and heparin chromatography. CTD kinase was purified through the Mono Q chromatography step. Both enzymes were dialysed against 60 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 0.1 mM DTT, and 10% glycerol. The reactions were carried out in dialysis buffer minus glycerol with 10 μ M [γ -³²P]ATP at a specific activity of 50 Ci mmol⁻¹ for 30 min at 30 °C. Reactions were stopped by addition of SDS sample buffer with 20 mM EDTA and boiled for 3 min. Samples were run on a 7% polyacrylamide gel, silver-stained using the method of Morrissey⁴¹, dried on Whatman DE81 paper, and autoradiographed. *b*, Two-dimensional paper electrophoretic determination of phosphoamino-acid content of hepta-6. The positions of the phosphoamino-acid markers are indicated. Hepta-6 was incubated with CTD kinase in the presence of 60 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 0.1 mM DTT, 20 μ M [γ -³²P]ATP at a specific activity of 10 Ci mmol⁻¹ at 30 °C for 4 hours. The reaction was stopped by addition of EDTA to 20 mM, and labelled peptide was purified on a 5 mm $\times$ 1.5 m Biogel P2 column run in 1M acetic acid. The labelled peptide was dried, resuspended in 6M HCl, and subjected to acid hydrolysis for 1 h at 100 °C (ref. 43). The hydrolysate was then dried, resuspended in formic acid/acetic acid/H₂O (1:10:89) pH 1.9, and subjected to two-dimensional paper electrophoresis⁴⁴. The plate was dried and stained with 1% ninhydrin in acetone and autoradiographed to reveal the labelled amino acid. *c*, SDS-PAGE of kinase reactions. Reactions run in lanes 1–3 contained CTD kinase, lanes 4–6 contained casein kinase II, and lanes 7–9 contained the catalytic subunit of cAMP kinase. Substrates in these reactions were: 0.5 mg ml⁻¹ hepta-4 (lanes 1, 4 and 7), 5 mg ml⁻¹ histone (Sigma, type II-AS; lanes 2, 5 and 8), and 5 mg ml⁻¹ casein (Sigma, dephosphorylated; lanes 3, 6 and 9). The positions of peptide (P), casein (C) and core histones (H) are indicated at the sides. CTD kinase and casein kinase were incubated in 60 mM KCl, 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 0.1 mM DTT, 200 μ M [γ -³²P]ATP at a specific activity of 100 mCi mmol⁻¹ with the designated substrates. Cyclic AMP kinase was incubated in 37.5 mM potassium phosphate, pH 6.0, 13.3 mM magnesium acetate, 200 μ M [γ -³²P]ATP at a specific activity of 100 mCi mmol⁻¹ with the designated substrates. Reactions were incubated for 30 min at 30 °C, stopped by the addition of SDS sample buffer with



20 mM EDTA and boiled for 3 min. The samples were electrophoresed on a 15% discontinuously buffered polyacrylamide gel⁴⁰. Sample loadings represent approximately equivalent amounts of activity against the principle substrate to achieve equal exposures on the gel. The gel was fixed in 10% TCA, 10% acetic acid, and 30% methanol, dried on Whatman DE81 paper, and autoradiographed.

FIG. 4 Comparisons of p34 peptide sequences and the amino-acid sequences of *cdc2* homologues. a, Three peptide sequences (TP14, TP16 and TP21) are shown above the cDNA sequences. Shaded characters indicate differences between the mouse sequence and the sequences of human *cdc2* (ref. 16), *S. pombe* *cdc2* (ref. 17), *S. cerevisiae* *CDC28* (ref. 18), and a partial sequence of a related human kinase (PSK-J3)¹⁹ sequences. CTD kinase was purified through the phenyl-Superose chromatography step. *In situ* tryptic digestion following the method of Aebersold⁴⁵ with the following modifications. CTD kinase (~350 pmol) was reduced and carboxamidomethylated in 10 mM Tris-HCl pH 8.0, 1% SDS, and 10 mM DTT for 60 min at 60 °C; followed by incubation with 4 µg ml⁻¹ iodoacetamide for 30 min at room temperature⁴⁵. The sample was precipitated in nine volumes ethanol at -20 °C overnight and pelleted by centrifugation. The pellet was resuspended in SDS sample buffer and run on a 10% discontinuously buffered SDS polyacrylamide gel⁴⁰. Proteins were transferred onto nitrocellulose by electroblotting for 1 h at 450 mA in 12.5 mM Tris, 96 mM Glycine, 20% methanol, and 0.005% SDS using an Idea Scientific Genie Blotter. The band of interest was visualized by Ponceau-S staining, and cut from the filter. The strip was cut into ~2 × 1.5-mm square pieces and treated with PVP-40 as described¹⁵. The filter pieces were then washed with 100 mM Tris-HCl pH 8.0 in 5% acetonitrile, and incubated overnight in 250 µl of that buffer with two additions of 0.25 µg TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone) treated trypsin (Cooper Biomedical). The released tryptic peptides were separated on reverse-phase HPLC using a Hewlett-Packard 1090 with a Brownlee Aquapore BU-300 (2.1 × 30 mm) column. A multiphasic gradient using 0.1% TFA in water as buffer A and acetonitrile/2-propanol (2:1) with 0.1% TFA as buffer B was run at 0.2 ml min⁻¹ with an oven temperature

MOUSE CDC2	MEDYIKIEKIGEGTYGVVYK	RHRVTGOIVAMKKIRLESEEEGVPSTAIRISLLKEL	58
HUMAN CDC2	MEDVTKIEKIGEGTYGVVYK	RHKTTGOVAMKKIRLESEEEGVPSTAIRISLLKEL	58
S POM CDC2	MENVQKVEKIGEGTYGVVYK	RHKLSGRIAMKKIRLESEEGVPSTAIRISLLKEY	58
S CER CDC28	HSSELANVVKLEKVGEGTYGVVYKALDRFGGQGRVYALKIRLESEDEGVPSTAIRISLLKEL		65

MOUSE CDC2	RHPNIVSLQDVLMDIS	RLYLIFEFLSMDLKKYLDISIP	PGOFMDSSTVKSYLHOIMOGI	116
HUMAN CDC2	RHPNIVSLQDVLMDIS	RLYLIFEFLSMDLKKYLDISIP	PGOYMDSSLVKSYLYOILQGI	116
S POM CDC2	NDENNRSNCGVRLDELHAES	KLYLVFEFLDMDLKKYMDRIS	ETGATSLDPRLLVQKFTYOLVNGV	122
S CER CDC28	KDDNIVRLDYIYHSDAHKLYLVFEFLDMDLKKYHDEGIP		KDOPLGADIVKFKFMHOLCKGI	124
HU PSK-J3		KYTLVFEHYDQDLRTYLDKAP	PPGLPAETIKDLHROFLRGL	41

TP14

MOUSE CDC2	VFCHSRRLVLRDLKPNLLIDDKGTIKLADFLARAFGIP	IRVYTHEVVTWYRSPEVLLGSARY	181
HUMAN CDC2	VFCHSRRLVLRDLKPNLLIDDKGTIKLADFLARAFGIP	IRVYTHEVVTWYRSPEVLLGSARY	181
S POM CDC2	NFCHSRRLVLRDLKPNLLIDDKGTIKLADFLARAFGIP	IRVYTHEVVTWYRSPEVLLGSARY	181
S CER CDC28	AYCHSRRLVLRDLKPNLLIDDKGTIKLADFLARAFGIP	IRVYTHEVVTWYRSPEVLLGSARY	181
HU PSK-J3	DFLHANCIVLRDLKPNLLIDDKGTIKLADFLARAFGIP	IRVYTHEVVTWYRSPEVLLGSARY	181

TP16

MOUSE CDC2	STPVDIWSIGTIFAELATKKPLFHGDSEIDOLFRI	FRALGTPNNEVWPEVESLQDYKNTFPKWP	246
HUMAN CDC2	STPVDIWSIGTIFAELATKKPLFHGDSEIDOLFRI	FRALGTPNNEVWPEVESLQDYKNTFPKWP	246
S POM CDC2	STGVDIWSVGCIFAEMIRRSPLFGDSEIDOLFRI	FQVLTGTPNNEVWPEVESLQDYKNTFPKWP	252
S CER CDC28	STGVDIWSIGTIFAEMCRNRPISFGDSEIDOLFRI	FRVLTGTPNNEVWPEVESLQDYKNTFPKWP	254

TP21

MOUSE CDC2	GSLSHYKVNLDENCLDFLSKMLVYDPAKRISGKMA	LKHYPYFDLNDNQIKKM	298
HUMAN CDC2	GSLSHYKVNLDENCLDFLSKMLVYDPAKRISGKMA	LKHYPYFDLNDNQIKKM	298
S POM CDC2	MDLHKVYPNGEEDAIELLSAMLVYDPAHRISAKRAL	QOQNYLRDFH	298
S CER CDC28	KDLSQVYPSLDPRGILLDLKLLAYDPINRISARRAA	THPYFOES	297

of 45° C while monitoring at 230 and 275 nm. Fractions containing peptides were manually collected in siliconized Eppendorf tubes and frozen at -70° C. Peptide sequencing was performed using an Applied Biosystems model 470A gas-phase sequencer and model 120PTH analyser. The peptide sequences shown were obtained at the 10–25 pmol range. The mouse *cdc2* cDNA was cloned using an oligonucleotide based on the human sequence from a cDNA library prepared from mouse 3T3 cell poly(A)⁺ RNA obtained 8–10 hours after serum stimulation of resting cells.

Cdc2 and cell-cycle control

Cell division cycle (*cdc*) genes were first defined in yeast by mutations that block cells at different points in the cell cycle. One class of these mutations arrest cells in G1 phase, at a point designated 'Start', when cells become committed to the mitotic cell cycle. One of the first genes identified by such mutations was the *S. cerevisiae* gene *CDC28* (ref. 22). Similar mutations have been described in the fission yeast *S. pombe* where the homologue of the *CDC28* gene has been identified as the *cdc2* gene²³. The *S. pombe* *cdc2* gene product is required in G1, at the 'Start' point of the cell cycle and, later in the same cycle, for mitosis²⁴. *Cdc2* and *CDC28* are extremely well conserved¹⁸ and can genetically complement one another²³. These genes encode 34K proteins that contain sequences in common with other protein kinases^{16–19}. *Cdc2*-like genes are highly conserved, both structurally and functionally, among a number of species.

TABLE 1 Immunoprecipitation of CTD kinase activity with anti-*cdc2* antiserum

		Anti- <i>cdc2</i> serum	Control serum
Purified CTD kinase	pellet	56%	1%
	supernatant	44%	99%
Nuclear extract	pellet	80%	3%
	supernatant	20%	97%

Rabbit antiserum raised against the C-terminal seven amino acids of human *cdc2* was used in immunoprecipitation reactions as described by Draetta and Beach²¹. Control serum was obtained from a rabbit that had not been injected with *cdc2*. CTD kinase assays were performed as described in the legend to Fig. 2. No loss in total activity was observed when CTD kinase-containing samples were incubated with either the anti-*cdc2* serum or the control serum. Results are expressed as the percentage of the total activity incubated with the antiserum.

Indeed, the human *cdc2*⁺ gene can complement a *cdc2* mutation in *S. pombe*¹⁶. The extremely conserved nature of this protein suggests that it performs fundamental functions in the cell cycle of all eukaryotic cells.

The molecular basis of p34 function is thought to be protein phosphorylation, although the identity of the *in vivo* substrates is unknown. A series of recent experiments have led to the idea that the cyclic nature of p34 function arises from assembly and disassembly of heterotypic multiprotein complexes containing p34. In this view, p34 kinase activity is enhanced by association with other subunits. In budding yeast, a complex of *M_r* 160K containing p34 accumulates during G1 and may be necessary for the G1-S transition²⁵. This complex phosphorylates p40 (another protein in the complex), histone H1, and presumably other proteins. It has been proposed that phosphorylation of p40 leads to disassembly of the 160K complex and loss of p34 kinase activity as cells enter S-phase²⁵; p34 has also been implicated in the G2-M transition. In these cases p34 forms complexes with cyclins—proteins that accumulate during interphase and are degraded at the end of mitosis^{26–28}. The formation of these complexes reaches a maximum in metaphase and coincides with the presence of M-phase-promoting factor (MPF) activity and M-phase-specific histone H1 kinase activity^{29–32}. The kinase activity of this complex increases 70-fold as cells move from G1 to mitosis and this increase is thought to trigger a chain of events that drives the interphase nucleus into mitosis. At the end of mitosis this complex dissociates and kinase activity is lost^{26,33}. Although the factors that control these cyclic assembly/disassembly processes are unknown, p34 is multiply phosphorylated in a cyclic fashion³³ and this may play some role in dictating p34 subunit interactions and kinase activity.

How is CTD kinase related to previously described p34-containing complexes? Several observations suggest that the CTD kinase is not equivalent to mammalian MPF/M-phase specific histone H1 kinase. First, CTD kinase does not phos-

phorylate histone or casein efficiently (Fig. 3c). Second, the largest subunit of the CTD kinase complex is smaller (58K) than the 62K subunit of HeLa MPF. Finally, we have recently detected CTD kinase activity in elutriated G1-phase cells (E. Barron, L.J.C. and J.C., unpublished observation) which is inconsistent with the observed loss of MPF/M-phase-specific histone H1 kinase activity after mitosis²¹. Whether CTD kinase is the p34 activity required in G1 is not known. It seems likely, however, that p34 can participate in a number of different complexes. In budding yeast only a small fraction of p34 is in the 160K complex during G1 (ref. 25). When anti-p34 antibodies are used to precipitate p34-containing complexes from HeLa cell extracts, a number of bands in the 40–60K range are detected³³. Thus, CTD kinase may be one of many p34 kinase activities. Transient association of p34 with a set of different subunits may allow for changes in the cell-cycle timing of its activity and substrate specificity.

CTD kinase and transcription

The greatest difficulty in establishing the role of CTD phosphorylation in transcription is that the mechanism of action of the CTD is unknown. One of the proposed functions of the CTD is to form specific contacts with transcription factors bound to *cis*-acting sites upstream of RNAP II-transcribed genes^{1,4}. The unphosphorylated form of the CTD may interact with transcriptional activator proteins perhaps through the formation of multiple hydrogen bonds between hydroxyl-containing side chains in the CTD and acidic residues in the activator proteins³⁴. This interaction could serve either as a recognition function or, alternatively, to stabilize specific interactions between the RNA polymerase core and general transcription factors bound close to the promoter. CTD kinase could then act to release RNAP II from pre-initiation complexes through phosphorylation of residues in the CTD; addition of phosphate to residues in the CTD serving to disrupt transcription factor–CTD interactions and allowing RNAP II to enter the elongation phase. This CTD kinase-facilitated 'release' mechanism is consistent with the observation that ATP hydrolysis is required during RNAP II transcription initiation at a stage following formation of the pre-initiation complex but before elongation of the nascent chain^{35,36}.

A second possible role for the CTD is to remove chromatin proteins from the path of transcribing RNAP II (ref. 1). In this model, the ability of the CTD to interact with basic proteins, such as histones, would be a function of the level of phosphorylation. Thus, the more highly phosphorylated the CTD is, the greater the rate of RNAP II transcription would be.

The identification of *cdc2* as a component of a protein kinase that phosphorylates RNAP II suggests that some aspects of cell-cycle control may take place through modulation of the level of phosphorylation of the CTD. Pulse-labelling experiments have shown that transcription in mammalian tissue culture cells is relatively constant through the cell cycle except for a brief period of arrest during mitosis³⁷. The function of *cdc2* may be causally related to the resumption of transcription in G1 phase in a variety of ways. One possibility is that phosphorylation/dephosphorylation of the CTD is a switch that is responsible for the arrest of transcription during M-phase. Arnt *et al.*³⁸ have recently identified a phosphoprotein phosphatase that is involved in regulating transcription in yeast. Such a phosphatase might act during mitosis to dephosphorylate the CTD and shut off transcription, whereas CTD kinase activity during G1 phase would activate RNAP II. The arrest of *cdc2* mutant cells in G1 phase would, therefore, be explained by a reduction in the level of CTD phosphorylation. This mechanism need not apply to transcription of all genes, but may pertain to some genes that are critical for cell-cycle control.

Summary

In the present study we report the isolation of a protein kinase that phosphorylates the CTD of RNAP II *in vitro*. This kinase, designated CTD kinase, phosphorylates one or more serine residues in the repeated CTD heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Although the role of this phosphorylation is unknown, the availability of purified CTD kinase will now enable direct tests of its function in transcription reactions *in vitro*. Furthermore, the identification of *cdc2* as a component of this protein kinase offers, for the first time, a genetic approach to the study of RNAP II phosphorylation. Our observation that the gene product of a cell-cycle control gene is a component of a protein kinase that phosphorylates RNAP II prompts us to take a new look at the cell-cycle regulation of transcription. □

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Comparison of oceanic and continental sources of non-sea-salt sulphate over the Pacific Ocean

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THE absence of long-term systematic measurements of sulphur species over the oceans has made it difficult to estimate the relative importance of what are believed to be the two major sources of non-sea-salt (n.s.s.) sulphate: reduced sulphur gases produced by biological activity in the oceans and sulphur compounds derived from continental anthropogenic sources. Here we summarize the results of aerosol sulphate measurements made over five- to seven-year periods from 1981 to 1987 at island stations in the Pacific Ocean (Fig. 1). The mean methanesulphonate (MSA)/n.s.s. SO_4^{2-} mass ratio, 0.065, at the relatively pristine sites on Fanning and American Samoa is believed to result solely from the atmospheric oxidation of reduced sulphur gases, primarily dimethylsulphide (DMS), emitted from the ocean. Estimates based on this ratio and mean MSA concentrations indicate that the biogenic source accounts for ~80% of the annual average n.s.s. SO_4^{2-} over the mid-latitude North Pacific. Thus, if n.s.s. SO_4^{2-} is the primary source of cloud condensation nuclei over the ocean, then the nuclei concentrations over the Pacific appear to be largely controlled by biological sources in the ocean.

Recently, Charlson *et al.*¹ hypothesized that oceanic phytoplankton might control the Earth's albedo and climate via variations in the production of DMS, which is emitted into the atmosphere and subsequently oxidized to SO_4^{2-} . SO_4^{2-} readily forms particles that interact strongly with solar and terrestrial radiation and which, as cloud condensation nuclei, may play significant roles in defining cloud albedo, surface cloud coverage and precipitation patterns. Schwartz², however, has challenged this hypothesis, arguing that even the large increases in SO_2 emissions from anthropogenic sources in the Northern Hemisphere have had no discernible effect on the cloud component of the satellite-derived planetary albedo or on the 100-year temperature records. Hence, he argues, variations in the DMS flux from the oceans should have no significant effect.

However, the assessment of climate effects is complicated by

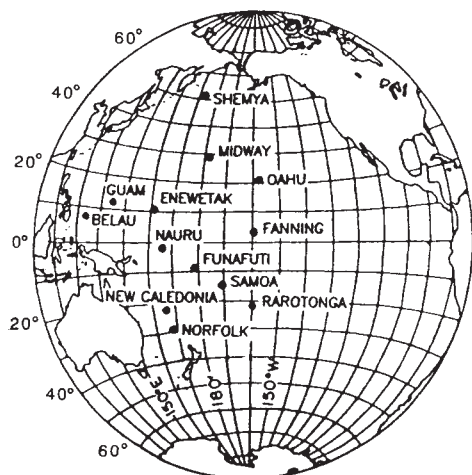


FIG. 1 Locations of the Pacific Ocean sampling stations used in the Sea-Air Exchange (SEAREX) Program.

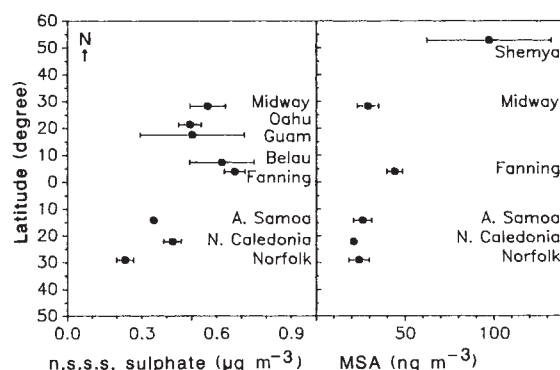


FIG. 2 Latitudinal variations in the mean concentrations of n.s.s. sulphate and MSA over the Pacific Ocean. The horizontal bars indicate the 95% confidence limits for the means. The n.s.s. sulphate results are based on weekly samples collected during 1981–87 at Midway and Oahu, 1981–82 at Guam and Belau, 1981–86 at Fanning, 1983–87 at American Samoa and Norfolk Island, and 1983–85 at New Caledonia. Samples analysed for MSA were those from 1981–82 over the North Pacific and 1983–84 over the South Pacific.

the non-uniform distributions of the major n.s.s. SO_4^{2-} sources. Some regions are expected to be dominated by anthropogenic sources and others by natural sources. Because ancillary factors (for example, meteorology, precipitation, surface albedo and solar flux) can vary seasonally, temporal variations in the relative strengths of natural and anthropogenic sources within a given region are also important. For example, the Arctic is ~20–40 times more polluted during the winter than during the summer³. Because of the much lower levels of solar radiation and more stable stratification of the Arctic troposphere during the winter, Arctic haze has substantially less impact on the Northern Hemisphere radiation budget and albedo than it would if it occurred during the summer.

Here we characterize the temporal and areal variability of particulate sulphate and MSA over the Pacific Ocean. Weekly samples were collected on 20 × 25 cm Whatman-41 filters at an air-flow rate of 1.1 m³ min⁻¹ with efficiencies >90% for n.s.s. SO_4^{2-} and MSA (ref. 5). To minimize contamination from local island sources, automatic controllers were used to turn the sampling pumps on only when the winds were off the ocean and at speeds >1 m s⁻¹.

SO_4^{2-} and MSA concentrations in aqueous extracts of the filters were determined to within ±5% by ion chromatography and sodium to within ±2% by flame atomic absorption⁴. The filter blank for MSA was below our detection limit; that for SO_4^{2-} averaged 28.4 μg per filter (sample standard deviation $s = 9.5$). We computed n.s.s. SO_4^{2-} by assuming that the seawater-derived SO_4^{2-} is equal to 0.252 times the Na^+ concentration. The absolute errors for measurements of n.s.s. SO_4^{2-} are equal to $\pm(0.05 \text{ SO}_4^{2-} + 0.01 \text{ Na}^+)$ and were usually <0.1 μg m⁻³. Samples with sea-salt Na^+ concentrations greater than 5 μg m⁻³ (essentially all of those at Shemya, Enewetak, Nauru, Rarotonga and Funafuti) are not considered here because of the unacceptably large errors in n.s.s. SO_4^{2-} .

The mean concentrations of both n.s.s. SO_4^{2-} and MSA exhibit substantial spatial variations (Fig. 2). The mean concentrations of n.s.s. SO_4^{2-} range from 0.23 μg m⁻³ at Norfolk Island to 0.67 μg m⁻³ at Fanning Island. As MSA in marine air is derived almost exclusively from the oxidation of DMS, the large-scale spatial variations in MSA are, as expected, in general agreement with those of the DMS concentrations that have been measured in the underlying oceans^{3,6}. The highest mean MSA concentration, 0.097 μg m⁻³, occurs at Shemya in the high-latitude North Pacific where DMS concentrations average 2.4 nmol l⁻¹. A relatively high mean MSA concentration, 0.044 μg m⁻³, also occurs at Fanning near the equatorial divergence, an area of high

biological productivity and mean DMS levels of 3.8 nmol l^{-1} . In the oligotrophic regions represented by the remaining stations, the mean MSA concentrations vary over the narrow range from ~ 0.02 to $0.03 \mu\text{g m}^{-3}$. Mean DMS concentrations in these regions range from 1.4 to 1.7 nmol l^{-1} .

We suggest that the n.s.s. SO_4^{2-} at both American Samoa and Fanning Island originates almost exclusively from the oxidation of biogenic sulphur gases emitted from the ocean. Mineral aerosol and trace-metal concentrations at American Samoa are among the lowest measured anywhere, including the South Pole^{7,8}, suggesting that continental sources have a minimal impact on aerosol chemistry here. The lowest mean n.s.s. SO_4^{2-} concentration ($0.28 \mu\text{g m}^{-3}$) occurs during April and May. During September and October, the concentrations are ~ 1.6 times higher, $0.45 \mu\text{g m}^{-3}$. Although there is no distinct seasonal cycle indicated by the limited MSA data, the MSA/n.s.s. SO_4^{2-} ratio is consistently ~ 0.065 in all the samples analysed⁹. Although Fanning is sometimes affected by the transport of dust from Asia during the months of February–May^{7,10}, these rare events have no pronounced effect on the n.s.s. SO_4^{2-} concentration, which is essentially constant throughout the year (Fig. 3). The absence of a distinct seasonal cycle in MSA is consistent with the reasonably constant surface-water DMS concentrations in this region⁷. As at Samoa, the mean MSA/n.s.s. SO_4^{2-} ratio at Fanning is ~ 0.065 (ref. 9). For samples collected at Fanning and Samoa and analysed for MSA, the mean MSA/n.s.s. SO_4^{2-} ratio is 0.065 ± 0.005 ($N = 51$, $s = 0.016$, $\pm$ indicates the 95% confidence limits of the mean).

That n.s.s. SO_4^{2-} at Samoa and Fanning is derived almost exclusively from biogenic sulphur emissions is not unreasonable. The estimated DMS emission fluxes and the estimated n.s.s. SO_4^{2-} deposition rates are comparable at both locations. Based on the mean n.s.s. SO_4^{2-} concentrations, a dry deposition velocity of 0.05 cm s^{-1} (ref. 11), a washout ratio of $0.36 (\mu\text{g ml}^{-1}$

rain)/($\mu\text{g m}^{-3}$ air) (ref. 12), and the average rainfall¹³, the n.s.s. SO_4^{2-} deposition rates are estimated to be 370 and $980 \mu\text{g SO}_4^{2-} \text{ m}^{-2} \text{ d}^{-1}$, respectively. Assuming that DMS oxidation accounts for 87% of the biogenic n.s.s. SO_4^{2-} (ref. 14) and 100% of the MSA, the required DMS emissions are 3.6 and $9.5 \mu\text{mol m}^{-2} \text{ d}^{-1}$, respectively. Based on the data presented by Bates *et al.*⁶, DMS emissions at the two sites are estimated to be ~ 2.9 and $6.8 \mu\text{mol m}^{-2} \text{ d}^{-1}$, respectively. Because of the large (probably factors of 2) uncertainties in estimates of both the emission and deposition fluxes, the differences are certainly not significant; the estimates only serve to indicate that there are no gross disparities.

In the unpolluted marine atmosphere, DMS is oxidized primarily by reaction with OH radicals to MSA (which is stable) and SO_2 (which is further oxidized to sulphate). As MSA and n.s.s. SO_4^{2-} have similar physical and chemical properties, they should be subject to similar removal mechanisms and rates. The reasonably consistent MSA/n.s.s. SO_4^{2-} mass ratio of 0.065 at Fanning and American Samoa may provide the basis for estimating the input of n.s.s. SO_4^{2-} from marine biogenic sources at other sites. Regression analyses indicate that a similar ratio occurs at Barbados, West Indies, when the continental contribution to n.s.s. SO_4^{2-} is not significant⁴. Recent kinetic studies¹⁵ indicate that the relative importance of hydrogen abstraction versus OH addition in the reaction of DMS with OH is strongly temperature-dependent. Unfortunately, it is still not clear how much of the MSA and SO_2 products are associated with each of the two pathways¹⁵, and, hence, how this temperature dependence affects the natural MSA/n.s.s. SO_4^{2-} ratio. At Fanning, Samoa and Barbados, the monthly mean air temperatures are within the range from 25 to $\sim 27^\circ\text{C}$ throughout the year.

Over the mid-latitude North Pacific, seasonally higher concentrations of n.s.s. SO_4^{2-} occur during the late winter and spring^{16,17}, corresponding to the period of major dust transport from Asia^{7,10}. The major impact of Asian material is most apparent at Midway, where the seasonal cycle of n.s.s. SO_4^{2-} (Fig. 3) is strikingly similar to that of mineral dust. A similar seasonal cycle is observed for aerosol nitrate¹⁸. During the five months of the year with the lowest dust levels (July–September, December and January), the mean n.s.s. SO_4^{2-} concentration, ($0.37 \mu\text{g m}^{-3}$) is a factor 2.5 lower than that during the spring peak from March to May, ($0.93 \mu\text{g m}^{-3}$). The biogenic source also exhibits a significant seasonal cycle. The mean MSA concentration from April to September, ($28\text{--}33 \text{ ng m}^{-3}$) is a factor of about three higher than that during January and February ($10\text{--}12 \text{ ng m}^{-3}$). Seasonal variations in surface-water DMS concentrations of the sub-tropical North Pacific are also a factor of about three⁶.

If we assume that a MSA/ SO_4^{2-} ratio of 0.065 is applicable for the marine biogenic source at Midway, this source supplies an annual average of $\sim 0.45 \mu\text{g m}^{-3}$ of n.s.s. SO_4^{2-} or $\sim 80\%$ of the total average n.s.s. SO_4^{2-} . Clearly, on an annual basis, the marine source dominates. From July to September, the monthly mean temperatures at Midway ($25.5\text{--}26^\circ\text{C}$) are comparable to those at Samoa, Fanning and Barbados. The mean MSA/n.s.s. SO_4^{2-} ratio of 0.072 during this period indicates that the biogenic source overwhelmingly dominates during the summer. Continental sources appear to be most important during March, when they provide $\sim 60\%$ of n.s.s. SO_4^{2-} . The percentage from continental sources would be underestimated for the winter months if the MSA/ SO_4^{2-} product ratio increases substantially with decreasing temperature; however, the monthly mean temperature at Midway never drops below 18°C . The relatively narrow spread in mean MSA and DMS concentrations in oligotrophic areas suggests that a comparable biogenic source probably exists for Oahu; if so, it would yield $\sim 90\%$ of the mean n.s.s. SO_4^{2-} . Andreae *et al.*¹⁹ have previously concluded that the biogenic source dominates in the marine boundary layer over the north-east Pacific even when the Asian source is evident in the free troposphere.

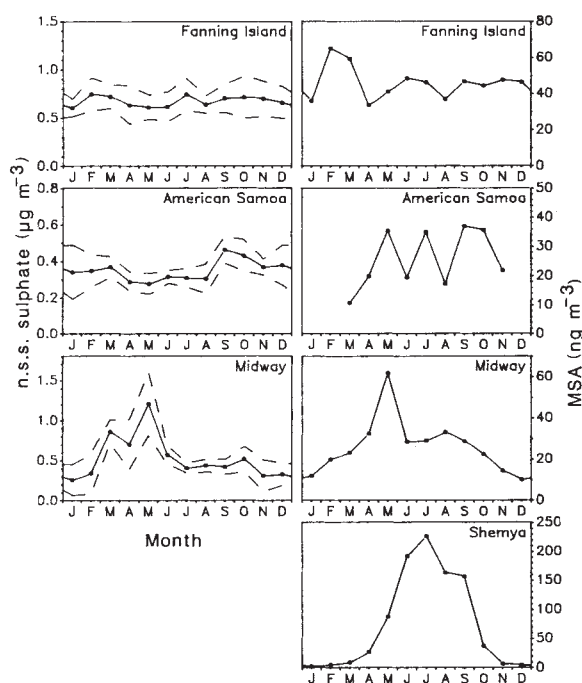


FIG. 3 Seasonal cycles of n.s.s. sulphate and MSA in the atmospheric boundary layer at Fanning Island, American Samoa, Midway Island and Shemya. The concentrations of n.s.s. sulphate are not shown for Shemya, where their errors were unacceptably large as a consequence of high sea-salt concentrations. The solid lines connect the monthly mean concentrations. The dashed lines in the n.s.s. sulphate plots indicate the upper and lower 95% confidence bounds of the monthly means. See Fig. 2 legend for periods covered by the data.

At Shemya, MSA exhibits not only its highest mean concentration but also its most dramatic seasonal cycle. From November to March, the concentrations average $<6 \text{ ng m}^{-3}$ compared to an average of $>150 \text{ ng m}^{-3}$ from June to September. Equally substantial cycles have been reported for the gas-phase precursor, DMS, in the surface waters of the Arctic and sub-Arctic⁶. Because we have no n.s.s. SO_4^{2-} data for Shemya, it is not possible to quantify the relative inputs from continental and oceanic sources. Continental sources, however, should clearly dominate during the winter, when Arctic haze and its associated high concentrations of pollutant SO_2 and n.s.s. SO_4^{2-} are likely to be transported into the region³ and, based on the MSA data, when the input from biological sources should be minimal. Despite our inability to assess accurately the n.s.s. SO_4^{2-} concentrations, we can state, based on the sea-salt concentrations and propagation of errors, that the summer mean is $\leq 2 \mu\text{g m}^{-3}$. The mean MSA levels indicate that the biogenic source could supply all of this n.s.s. SO_4^{2-} .

On a global basis, anthropogenic sulphur emissions are equal to, or greater than, all biogenic emissions and at least twice as great as oceanic reduced sulphur emissions². Despite the size of the anthropogenic sources, they appear to have only a small impact on aerosol composition over the North Pacific. If the Midway data are representative of the large area of the North Pacific that is known to be affected by Asian sources, then the continental fraction accounts for only $\sim 20\%$ of the n.s.s. SO_4^{2-} aerosol. The continental fraction is certainly expected to be higher near China and Japan; however, it should also be significantly lower in the more southerly region of the North Pacific trade-wind regime. Although anthropogenic sources certainly dominate in industrialized areas and in downwind regions, their

dominance is neither as pervasive nor as large as Schwartz² implies. For example, only $\sim 20\text{--}25\%$ of North American combustion emissions are exported from the continent; most are deposited locally or regionally^{20,21}. Thus, the n.s.s. SO_4^{2-} concentrations over the Pacific appear to be primarily controlled by biological sources in the ocean. If, as has been suggested, this sulphur is indeed the major component of CCN, then these sources also control the CCN levels over this region. □

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Effect of continental sources on nitrate concentrations over the Pacific Ocean

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ESTIMATES of atmospheric nitrogen budgets, based largely on nitrate measurements, suggest that over half of the reactive nitrogen is derived from anthropogenic sources¹. As there are so few data for the oceans it is difficult to assess the impact of continental sources on the marine atmosphere. Here we present data from several years of continuous sampling in a Pacific island network. The lowest concentrations, $0.11 \mu\text{g m}^{-3}$ (ref. 2), were consistently obtained at three South Pacific stations, where the effect of continental sources is minimal³. By contrast, in the North Pacific, nitrate concentration was about three times greater and was co-seasonal with Asian dust transport, suggesting that much of the nitrate over the North Pacific is derived from continental sources^{3–5}. If we assume that the South Pacific nitrate values are representative of oceanic 'background' sources and that these background values are applicable to the North Pacific, then we conclude that continental sources, which are predominantly anthropogenic¹, are responsible for 40–70% of the atmospheric nitrate over this region.

The measurements were initiated to obtain a large-scale climatology of nitrate and other aerosol species over the Pacific Ocean. The sampling network was established in the North Pacific in 1981 as a part of the Sea–Air Exchange Program (SEAREX)⁶ and was extended in 1983 to the South Pacific (Fig. 1). Week-long bulk aerosol samples were collected from onshore

winds using $20 \times 25 \text{ cm}$ Whatman 41 filters. At a flow rate of $1.1 \text{ m}^3 \text{ min}^{-1}$ the filter efficiency was $>95\%$ (refs 6, 7). Nitrate was measured in aqueous filter extracts by ion chromatography to within $\pm 5\%$ (refs 2, 8). Sample size was generally at least 100 times greater than that of field filter blanks ($7.6 \mu\text{g}$ nitrate).

We report values for total nitrate (particulate nitrate plus gaseous HNO_3) because, in addition to aerosol, filters loaded with sea salt should collect gas-phase HNO_3 with essentially 100% efficiency^{9–12}. Estimates of the ratio of gas-phase nitrate to total nitrate in the near-surface marine boundary layer range from <0.1 (refs 13, 14) to ~ 0.3 (ref. 15).

The nitrate results are presented in Table 1 and shown in Fig. 2. The mean values range from 0.107 to $0.359 \mu\text{g m}^{-3}$. North Pacific nitrate concentrations are consistently and substantially

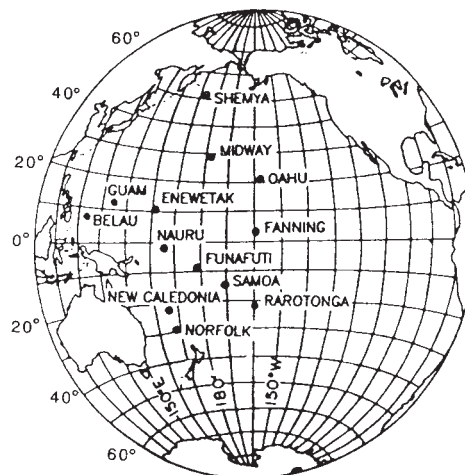


FIG. 1 The aerosol sampling network in the Pacific Ocean.

higher and more variable than those over the equatorial and central tropical South Pacific.

On the basis of Table 1 (and other factors discussed below) we divide the data into four groups: Funafuti (Tuvalu), American Samoa, Rarotonga (Cook Islands)-FASR; Shemya (Aleutian Islands), Midway and Oahu-SMO; Enewetak (Marshall Islands), Fanning (Christmas Islands) and Nauru-EFN; New Caledonia and Norfolk Island-NCNI. We exclude Guam and Belau because we believe that the samples are occasionally affected by local sources.

FASR. Nitrate concentrations are lowest in this central South Pacific group and there is no significant difference among them, the group mean being $0.113 \mu\text{g m}^{-3}$ (ref. 2). There is evidence of an annual cycle in nitrate concentration, most obviously at American Samoa (Fig. 3a) where the concentration in September–November is 1.4 times higher than March–May.

SMO. The highest nitrate concentrations occurred in the central North Pacific with the maximum at Oahu, $0.359 \mu\text{g m}^{-3}$ (refs 6, 16). There is a strong seasonal cycle at all three stations, as typified by the data from Midway (Fig. 3b) which shows a well-defined maximum in the spring. At Midway, the ratio of monthly mean maximum to minimum is 1.9, and at Oahu it is 2.2.

EFN. The equatorial stations constitute an intermediate group with annual means of 0.165 – $0.155 \mu\text{g m}^{-3}$. At Nauru and Fanning, which lie predominantly in the Southern Hemisphere circulation, there is no consistent seasonal pattern in nitrate concentrations. However at Enewetak, the seasonal cycle is the same as that at Midway, Oahu and Shemya.

NCNI. The mean concentrations are also in the intermediate range, comparable to EFN. However, in contrast to Fanning and Nauru, there is a strong seasonal cycle with the maximum in the austral summer and the minimum in the winter.

The temporal and areal variability of nitrate corresponds in a general way to that reported for mineral dust at these stations^{3–5,17}. The highest dust concentrations are observed in the central North Pacific and the lowest in the central South Pacific (Table 1). At SMO, the highest monthly mean dust concentrations occur in the spring^{4,5}, dropping by a factor of 20 in the summer. For example, at Midway, mean values for March are $2.2 \mu\text{g m}^{-3}$ whereas those for July are $0.097 \mu\text{g m}^{-3}$. This mineral aerosol is traced to dust storms in Asia, the dust being carried over the Pacific in the high-level westerlies¹⁸. Substantial concentrations of Asian dust are also measured at stations that lie deep in the easterlies; for example, at Enewetak the annual dust cycle is identical to that at SMO although concentrations are considerably lower^{4,5}. Asian dust events occasionally penetrate the intertropical convergence zone. At

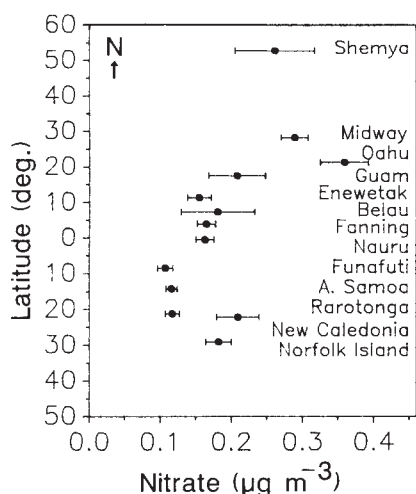


FIG. 2 Latitudinal distribution of the mean concentrations of nitrate over the Pacific as measured in the network¹⁶. The horizontal bars indicate the 95% confidence limits for the means.

TABLE 1 Nitrate and mineral aerosol data for the Pacific

Station	Nitrate aerosol*	Background ratio†	Mineral dust*
Shemya	0.261	2.37	0.860
Midway	0.289	2.63	0.906
Oahu	0.359	3.26	0.738
Guam	0.208	1.89	—
Enewetak	0.155	1.41	0.283
Belau	0.181	1.65	—
Fanning	0.165	1.50	0.107
Nauru	0.163	1.48	0.105
Funafuti	0.107	1.00	(0.211)‡
American Samoa	0.116	1.00	0.019
Rarotonga	0.117	1.00	(0.112)‡
New Caledonia	0.209	1.90	0.182
Norfolk Island	0.182	1.65	0.436

Nitrate data from ref. 16 and aerosol data from ref. 3.

* Annual mean ($\mu\text{g m}^{-3}$).

† Ratio of the mean annual nitrate concentration to that for Funafuti, American Samoa and Rarotonga.

‡ The mineral aerosol concentrations cited for Funafuti and Rarotonga are upper limits. It is believed that some samples may be contaminated by mineral sediment which is transported in spray droplets during heavy surf conditions in the vicinity of the sampler.

such times increased dust concentrations are observed at Fanning and Nauru; however, such episodes are rare and dust concentrations at these stations are generally quite low.

A seasonal cycle is also observed in dust concentrations at NCNI. Maximum concentrations occur in the austral summer and minimum in the winter. The dust is believed to be transported from sources in Australia³.

The lowest dust concentrations occur at FASR, the annual mean at American Samoa, $0.019 \mu\text{g m}^{-3}$, being by far the lowest in the network³. The Samoa mean is the lowest measured at any ocean site; it is even lower than that measured in the Antarctic polar regions^{19,20}.

Thus there appears to be a general correspondence between mineral dust concentrations and nitrate (Table 1). We observe the lowest dust and lowest nitrate concentrations at American Samoa; we observe the highest dust and highest nitrate concentrations at SMO. Furthermore, the seasonal variability of nitrate is most pronounced at those stations that show a large seasonal variability in dust concentration. This is most notable at the SMO stations, all of which are heavily affected by Asian sources; it is also quite noticeable at NCNI which is affected by Australian sources. Even at Enewetak, where nitrate concentrations are relatively low, there is a very strong seasonal nitrate cycle that is identical to that of Asian dust.

On the basis of the close correspondence of the dust and the nitrate at SMO and our knowledge of the meteorological conditions associated with dust transport^{18,21}, we conclude that Asia is the major source of the North Pacific nitrate. The latitudinal distribution of nitrate corresponds to the distribution of NO and NO₂ (NO_x) emissions from combustion sources²². If we consider all sources between the western boundary of the Pacific and 95° E (a region that includes the major sources in Asia, south-east Asia, Oceania and Australia/New Zealand) then 70% of the emissions will lie between 23° N and 47° N. Furthermore, 40% of the NO_x sources will lie between 31–39° N, the same latitude band in which the major Asian dust sources are found²¹.

In the summer, North American sources may also be important for Oahu and much of the tropical North Pacific^{23,24}. However, the impact of North American sources appears to be relatively low as the nitrate concentrations at Oahu are at a minimum this time of year. The low impact may be partly due to the fact that air-parcel trajectories at this time of year are confined primarily to the marine boundary layer^{18,21}.

By contrast, at FASR, the occurrence of low nitrate concentrations in conjunction with extremely low mineral dust (at American Samoa) suggests that these sites are minimally affected by continental sources. Thus, the nitrate in this region may well be representative of the natural oceanic background. Nitrate background concentrations of $\sim 0.1 \mu\text{g m}^{-3}$ are often observed in the records of other oceanic sites, even in the North Pacific. At Eniwetok, concentrations in this range are observed all summer long⁶. Similar concentrations are noted at times in the tropical North Atlantic at Barbados⁸ and more frequently in the Indian Ocean in association with long over-ocean trajectories from the Southern Hemisphere²⁵.

We can estimate the impact of continental nitrate over the North Pacific if we assume that the nitrate values measured in the equatorial and central South Pacific are representative of the oceanic 'background' and that these values are applicable to the central North Pacific. At Midway, the mean nitrate concentration during the spring peak is $0.40 \mu\text{g m}^{-3}$. Assuming a 'background' level of $0.11 \mu\text{g m}^{-3}$, the transport from Asia would account for $\sim 0.29 \mu\text{g m}^{-3}$ during this period. Thus, continental sources would appear to account for 73% of the nitrate in the spring. On the other hand, if the equatorial (EFN) mean ($0.16 \mu\text{g m}^{-3}$) is used for the background value, then the continental nitrate fraction at Midway in the spring would be 60%.

If we estimate the continental impact on an annual basis in the same way, then we obtain the following percentages: Shemya, 39–58%; Midway, 45–62%; Oahu, 55–69%. Thus, ~ 40 –70% of the nitrate over the central North Pacific appears to be derived from continental sources. By way of comparison, ~ 20 % of the aerosol excess sulphate in this region is believed

to come from continental sources²⁶. The difference between the nitrate and the sulphate continental impact transport fluxes is probably due to the fact that there are major biological sources of sulphur in the ocean but no equivalent sources for nitrate precursors.

We estimate that 1.6 Tg of nitrate-nitrogen are deposited annually to the North Pacific lying outside the coastal and equatorial regions (ref. 7; J.M.P. and D.L.S., unpublished data). Our estimate of the continental fraction of the nitrate aerosol (40–70%) would yield an annual deposit of 0.7–1.1 Tg N. Recently, Levy and Moxim²⁴ used a general circulation model to simulate the global distribution and deposition of fossil-fuel-derived reactive nitrogen. They estimate that 1.3 Tg N (30% of the global total of 4.5 Tg) is derived from Asian sources and deposited to the North Pacific. Our measurements indicate that this estimate is reasonably correct.

The Southern Hemisphere ocean does not appear to be substantially affected by anthropogenic sources in general or by Northern Hemisphere sources in particular. The large difference between North and South Pacific nitrate concentrations is taken as evidence that cross-equatorial transport is sharply suppressed by the intertropical convergence zone (ITCZ). ITCZ precipitation processes efficiently remove aerosol nitrate and its gaseous precursors²⁴. Northern Hemisphere air parcels (as indicated by sharply increased concentrations of gaseous pollutants such as CO_2 and methylchloroform²⁷) are often observed at American Samoa during March and April; however, nitrate concentrations during this time of year are actually at a minimum². Southern Hemisphere combustion sources can account for only ~ 10 % of the observed nitrate concentrations over the central South Pacific²⁴. It has been suggested that about half of the nitrate could be derived from stratospheric injection²⁸; indeed, the seasonal cycle of nitrate at American Samoa is similar to that for ozone²⁹ which is known to have a stratospheric source^{24,30}. However, concurrent measurements of aerosol nitrate with ^{210}Pb and ^7Be (ref. 2) suggest that a stratospheric source is not dominant. The source of the remaining nitrate and its precursors is unknown although biomass burning and oceanic sources are possible candidates². □

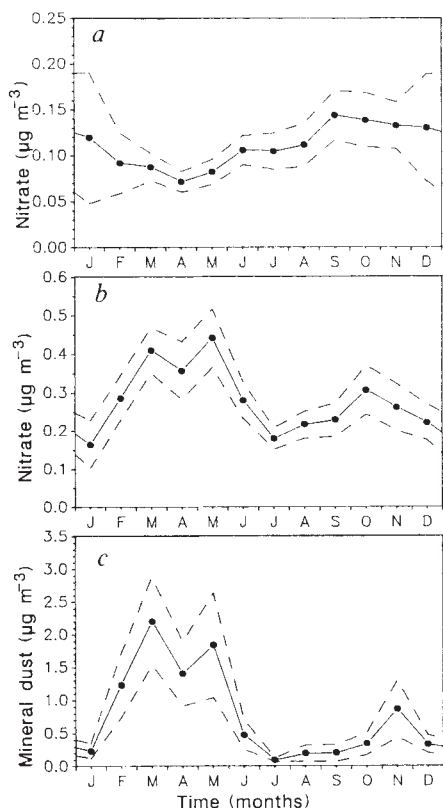


FIG. 3 Mean monthly concentrations showing the seasonal cycles of: a, nitrate at American Samoa¹⁶; b, nitrate at Midway¹⁶; c, mineral dust at Midway³. The solid lines connect the monthly mean concentrations. The dashed lines indicate the upper and lower 95% confidence bounds of the monthly means. The data cover the years 1983–87 for American Samoa and 1981–87 for Midway.

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Calculated fusion rates in isotopic hydrogen molecules

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COLD fusion occurs when two nuclei with very small relative energy tunnel through their mutual Coulomb barrier to initiate a nuclear reaction. The phenomenon is well studied in muon-catalysed fusion¹⁻⁴, where a relatively massive muon replaces an electron in a diatomic molecule of hydrogen isotopes, enhancing the binding and producing cold-fusion rates of $\sim 10^{12} \text{ s}^{-1}$. Cold fusion is also believed to occur as pycno-nuclear reactions in certain astrophysical environments⁵. Recent reports of cold fusion between hydrogen isotopes embedded in palladium⁶ and titanium⁷ have prompted us to reconsider previous estimates of the cold-fusion rates for free diatomic isotopic hydrogen molecules. In particular, we have calculated rates in diatomic hydrogen molecules of various isotopic composition. An accurate Born-Oppenheimer potential was used to calculate the ground-state wavefunctions. We find that the rate for d+d fusion is $3 \times 10^{-64} \text{ s}^{-1}$, some 10 orders of magnitude faster than a previous estimate. We also find that the rate for p+d fusion is 10^{-55} s^{-1} , which is larger than the d+d rate because of the enhanced tunnelling in the lighter system. Hypothetical enhancements of the electron mass by factors of 5-10 would be required to bring cold-fusion rates into the range of recently claimed observations.

Consider a free diatomic molecule composed of two hydrogen nuclei (which might be different isotopes). In the Born-Oppenheimer approximation the fusion rate Λ is proportional to the probability that the nuclei are very close together:

$$\Lambda = A|\Psi(\rho)|^2 \quad (1)$$

where Ψ is the normalized wavefunction describing their relative motion. The internuclear separation ρ is a typical distance at which nuclear interactions occur, approximately 10^{-14} m (10 fm).

The nuclear rate constant A for a given pair of nuclei is related to the low-energy behaviour of the corresponding fusion cross-section. If the variation of the cross-section $\sigma(E)$ with relative energy E is parameterized in terms of the usual S -factor,

$$\sigma(E) = \frac{S(E)}{E} e^{-2\pi\eta}, \quad \eta = \frac{e^2}{(2E\hbar^2/\mu)^{1/2}} \quad (2)$$

where μ is the reduced mass of the two nuclei and e is the charge on the electron, then

$$A = \frac{S(E=0)}{\mu c^2} \frac{c}{\pi\alpha} \quad (3)$$

with $\alpha = e^2/\hbar c \approx 1/137$. Table 1 shows the nuclear rate constants for five possible interactions between two hydrogen nuclei⁸.

We restrict ourselves only to s -wave nuclear motion (for which the fusion rate will be largest), so that the wavefunction can be written as

$$\Psi(r) = \frac{\psi(r)}{(4\pi)^{1/2}r} \quad (4)$$

with the normalization $\int_0^\infty \psi^2 dr = 1$. The radial wavefunction ψ then satisfies the Schrödinger equation

$$\left[-\frac{\hbar^2}{2\mu} \frac{d^2}{dr^2} + V(r) \right] \psi(r) = \epsilon \psi(r) \quad (5)$$

where ϵ is the eigenvalue for relative motion. Unless otherwise specified, we will hereafter work in atomic units ($e^2 = \hbar = m_e = 1$), so that all energies are measured in hartrees ($\approx 27.2 \text{ eV}$) and all distances are measured in Bohr radii ($a \approx 0.53 \times 10^{-8} \text{ cm}$).

Simple considerations determine the general features of the potential $V(r)$. If energies are measured relative to the energy of two isolated hydrogen atoms ($= -1$ in these units), V vanishes at large r . Furthermore, it must have a minimum of the depth and separation required to support the observed molecular bound states. At small separations, the electronic structure is that of the He atom with an energy of $V_0 = -1.9037$, so that

$$V(r \rightarrow 0) \rightarrow \frac{1}{r} + V_0 \quad (6)$$

An estimate of the suppression of the fusion rate by tunnelling is given by the barrier penetration factor obtained from the Wentzel-Kramers-Brillouin (WKB) approximation to equation (5):

$$B = \exp \left[-2 \int_0^{r_0} k(r) dr \right] \quad (7)$$

where the local wavenumber is $k(r) = [2\mu(V(r) - \epsilon)]^{1/2}$ and the integral extends to the classical turning point, r_0 .

To estimate the barrier penetration integral (7), we have taken for the diatomic molecular potential $V(r)$ the best available numerical calculation in the Born-Oppenheimer approximation, due to Kolos and Wolniewicz^{9,10} (K-W). For $1.1 < r < 3$ this potential is well approximated by the Morse potential

$$V(r) = 0.1745[e^{-2.08(r-1.4)} - 2e^{-1.04(r-1.4)}] \quad (8)$$

For smaller values of r we fitted the calculated values of $V - 1/r$ to a seven-term Lagrange interpolation formula. Numerically evaluating the integral using the exact d+d eigenvalue and turning point, we find

$$B = e^{-4.13\sqrt{\mu}} \quad (9)$$

The numerical coefficient of 4.13 is to be compared with the estimate of 3.0-3.3 made by Zeldovich and Gershtein²; the difference leads to a penetration factor that is 15-21 orders of magnitude smaller.

An accurate evaluation of the cold-fusion rates can be obtained by a direct numerical integration of the Schrödinger equation (5) with the K-W potential, as shown in the second column of Table 2. The nuclear radius was taken to be $\rho = 2 \times 10^{-4}$ ($\approx 10 \text{ fm}$). The numerical methods of ref. 11 were used, with the wavefunction being treated explicitly only for $r > 0.005$; the regular s -wave Coulomb function was used to extrapolate this solution to $r = \rho$.

The exact dependence of the barrier factor on reduced mass that we extract from these results is in good agreement with the WKB approximation (7), but is not consistent with refs 2 and 12. The estimate of the d+d fusion rate made in ref. 12 is too low by about 10 orders of magnitude, because in the calculation of the WKB penetration integrals an unshifted Coulomb potential was used at small separations (equivalent to our equation (6) with $V_0 = 0$). The effective energy with which the nuclei strike the Coulomb barrier is therefore lower than it should be, and hence the calculated fusion rate is smaller. We note that the p+d fusion rate is 8.5 orders of magnitude larger than the d+d

TABLE 1 Rate constants for fusion of hydrogen isotopes

Reaction	$S(E=0)$ (MeV barn)	A ($\text{cm}^3 \text{ s}^{-1}$)
$p+p \rightarrow {}^2\text{H} + e^+ + \nu_e$	3×10^{-25}	8×10^{-40}
$p+d \rightarrow {}^3\text{He} + \gamma$	2.5×10^{-7}	5.2×10^{-22}
$p+t \rightarrow {}^4\text{He} + \gamma$	2.6×10^{-6}	4.8×10^{-21}
$d+d \rightarrow {}^3\text{He} + n \oplus {}^3\text{H} + p$	1.1×10^{-1}	1.5×10^{-16}
$d+t \rightarrow {}^4\text{He} + n$	1.1×10^1	1.3×10^{-14}

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rate. Although the nuclear rate constant for $p+d$ is 5.5 orders of magnitude smaller than for $d+d$, the smaller reduced mass in the former case enhances the tunnelling probability more than enough to compensate for this. This same effect allows the weak $p+p$ fusion to compete favourably with the strong $d+t$ reaction.

We have also calculated fusion rates in other states of the hydrogen molecule. For example, the $p+d$ and $d+d$ fusion rates in the second vibrationally excited state are enhanced by 2.5 orders of magnitude relative to the ground-state values. Furthermore, the $d+d$ fusion rate in the ground state of the molecular ion was calculated using the exact Born-Oppenheimer potential tabulated in ref. 13 to be smaller than that of the molecular ground state by 6.5 orders of magnitude. For the general molecular-ion ground state, the coefficient 4.13 in equation (9) changes to 4.80.

It is interesting to ask by how much the internuclear separation must be decreased to reach the fusion rates claimed in refs 6 and 7. Although the precise answer depends on the details of the internuclear potential, a simple way of quantifying the problem is simply to change the radial scale. This is equivalent to endowing the electron with a larger mass m^* than it actually has, in which case the separation scales as m_e/m^* . (This enhanced mass should not be associated with any physical excitation in a solid material, as only the bare electron is relevant at the short length scales that are important here.) Accurate estimates can be made by numerical integration of the Schrödinger equation (5) with the K-W potential^{9,10} as shown in Table 2. For other values of m^* we have found that a very good fit is given by

$$\log_{10} \Lambda = 6.5 + \log_{10} (A/a^3) + 3 \log_{10} (\mu/M_n) - 79(\mu/M_n)^{1/2}(m_e/m^*)^{1/2} \quad (10)$$

where M_n is the nucleon mass and the variation with m^* is that given by equation (9). A mass enhancement of $m^* \approx 5m_e$ would be required to bring the cold-fusion rates into the range claimed in ref. 7. This is 2.5 times larger than the value calculated previously^{2,12}. An enhancement of $m^* \approx 10m_e$ is required by the results of ref. 6.

It is worth remarking on the validity of the Born-Oppenheimer approximation as used in this context. As there is a large difference between the potential and total energies in the classically forbidden region, one might naively expect a failure of the adiabatic assumption. However, more careful reflection suggests otherwise. Systematic corrections to the adiabatic approximation are possible by considering the full coupled-channels generalization of equation (5)¹⁴. The largest coupling terms are of order

$$\frac{1}{\mu} \left\langle n \left| \frac{\partial}{\partial r} \right| m \right\rangle \frac{d\Psi_m(r)}{dr} \approx \frac{k(r)}{\mu} \Psi_m(r) \quad (11)$$

where n, m label the electronic states, which we assume to vary on the scale of the Bohr radius. The local wavenumber at small distances is $k(r) \approx (2\mu/r)^{1/2}$. This term is to be compared with the effect of the diagonal potential at short distances, $\sim \Psi_m/r$, giving a correction to adiabaticity of the order of $(k/\mu)/(1/r) \approx (r/\mu)^{1/2} \ll 1$.

TABLE 2 Cold-fusion rates in isotopic hydrogen molecules

	$m^*/m_e=1$	2	5	10
$p+p$	-64.4	-48.0	-33.2	-25.6
$p+d$	-55.0	-36.0	-19.0	-10.4
$p+t$	-57.8	-37.7	-19.7	-10.5
$d+d$	-63.5	-40.4	-19.8	-9.1
$d+t$	-68.9	-43.5	-20.9	-9.4

Cold fusion rates are expressed as $\log_{10}$ of the rate in s^{-1} .

These calculations of the fusion of hydrogen nuclei embedded in a metal are only approximate. Screening by the electrons modifies the Born-Oppenheimer potential¹⁵. Moreover, fluctuations present in many-body situations might significantly enhance fusion rates¹⁶, although there are limits to the efficacy of this mechanism in equilibrium conditions¹⁷.

In summary, we find that the rate for $d+d$ fusion in the free hydrogen molecule ($\sim 3 \times 10^{-64} s^{-1}$) is 10 orders of magnitude larger than the most recent previous estimate¹², and that the rate for $p+d$ is faster still by 8 orders of magnitude. The latter remains true for rates slower than $\sim 3 \times 10^{-17} s^{-1}$ ($m^* \approx 6m_e$). Hence, if in the experiments of ref. 7 any nuclear process is occurring at all, the neutron-free $p+d$ reaction could be playing a role. We also find that hypothetical enhancements of the electron mass by factors of 5–10 are required to bring cold-fusion rates into the range of values claimed experimentally. However, we know of no plausible mechanism for achieving such enhancements.

After submission of this manuscript, we became aware of similar calculations by H. Picker¹⁸ that agree in part with our results. □

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Evidence for non-metallic nature of the BiO plane in $\text{Bi}_2\text{CaSr}_2\text{Cu}_2\text{O}_8$ from scanning tunnelling spectroscopy

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THE existence of Fermi-liquid electronic states in the high- T_c superconductor $\text{Bi}_2\text{CaSr}_2\text{Cu}_2\text{O}_8$ has been experimentally established by angle-resolved photoemission spectroscopy¹. The nature of these states has been studied by various high-energy spectroscopies^{1–5}, all of which indicate that the electronic states at the Fermi energy reside mainly in the oxygen $2p$ orbital with in-plane symmetry. The question remains as to which plane in the crystal, BiO or CuO_2 , provides the O $2p$ orbitals at the Fermi energy. This point is important in modelling the high- T_c mechanism,

because if the Fermi-liquid states were supplied by the BiO plane, which is not present in the copper oxide high- T_c superconductors, mechanisms based on the Fermi-liquid states would lose their direct experimental basis. Here we present results from scanning tunnelling spectroscopy (STS), combined with photoemission (PES) and inverse photoemission (IPES) spectroscopies, which show that the BiO planes are non-metallic and the O 2p orbitals in the CuO₂ planes form the Fermi-liquid states.

Band-structure calculations⁶⁻⁸ have predicted that a Bi 6p-O 2p band crosses the Fermi energy E_F , giving a substantial density of states at E_F . Although photoemission studies^{1,9-11} have resolved the Fermi-edge structure in Bi₂CaSr₂Cu₂O₈, the nature of the Fermi-edge states is controversial; they have been assigned by some to the BiO plane^{9,10} and by others to the CuO₂ plane^{1,11}. Photoemission spectroscopy alone cannot determine the location of the O 2p orbitals, because it probes a number of layers into the surface, and thus contains contributions from both BiO and CuO₂ planes. To differentiate these two possibilities, we have performed STS measurements on single-crystal Bi₂CaSr₂Cu₂O₈. STS is a spectroscopic extension of scanning tunnelling microscopy (STM)¹² which has been developed for studying atomic structure at surfaces in real space. The technique has proved to be useful for high-energy-resolution studies of the electronic structure of the uppermost atomic layer of a crystal¹³.

We used a single crystal of Bi₂CaSr₂Cu₂O₈ (of dimensions $5 \times 5 \times 0.5$ mm) grown by a KCl flux technique. Such crystals have a smooth plane along which cleavage is easy. A magnetic-susceptibility measurement showed that the crystal becomes superconducting at 85 K. The crystal was cleaved in the STM/STS spectrometer and was kept at room temperature under ultra-high vacuum ($<1 \times 10^{-9}$ torr) during measurements. The details of our STM/STS apparatus and experimental procedures have been described elsewhere¹⁴. We obtained a large-area STM image which shows a series of rows spaced by about 2.6 nm (Fig. 1); these rows correspond to the incommensurate superstructure along the b axis that is observed in the bulk crystal^{15,16}, thus indicating that the cleaved surface is a BiO plane. The fact that the same incommensurate superstructure appears on the cleaved surface as in the bulk indicates that there is a very weak interlayer interaction between BiO planes in bulk. Thus one would expect little difference between the electronic structure of a BiO plane on the surface, as probed by STS, and that of a BiO plane in the bulk.

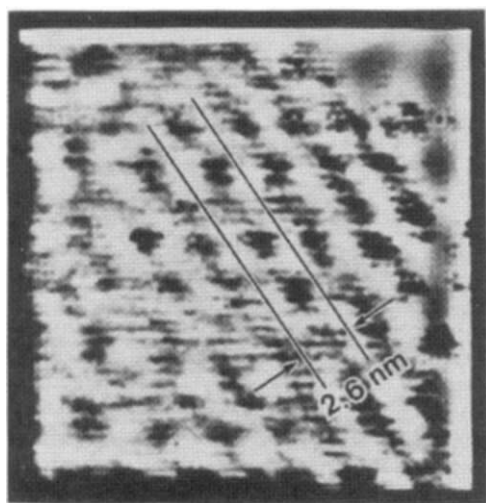


FIG. 1 STM image of a cleaved surface of a Bi₂CaSr₂Cu₂O₈ single crystal, measured with a current of 1 nA, a sample bias of +0.5 V and a scanning area of 23×23 nm. The series of rows spaced at 2.6 nm represents the incommensurate superstructure of a surface BiO plane.

Figure 2 shows representative $I-V$ and conductivity (dI/dV) curves obtained by averaging STS spectra over a grid of 128×128 points in the image of Fig. 1. The $I-V$ curve shows a flat region around 0 V sample bias, and the conductivity falls to zero in that region. This indicates that the density of states at E_F is negligibly small, suggesting that the BiO plane is non-metallic.

The conductivity curve does not necessarily represent the exact density of states, however, because dI/dV is proportional to the product of the density of states and the transmission factor between tip and sample¹⁷. Figure 3 shows the normalized conductivity curve $[(dI/dV)/(I/V)]$ obtained from the $I-V$ curve in Fig. 2. It has been shown that the normalized conductivity provides a measure of the density of states that is approximately independent of the tip-sample separation^{17,18}. Recently, however, Martensson and Feenstra¹⁹ found that, for a large surface band gap, calculations of the normalized conductivity

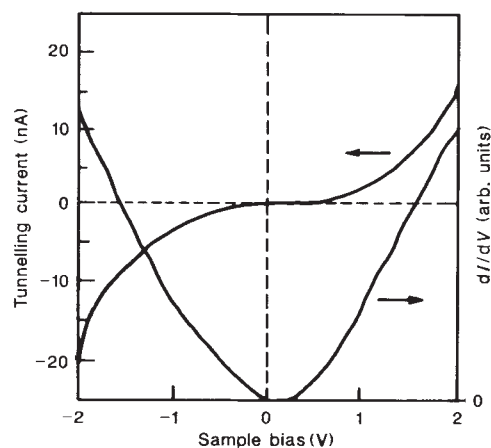


FIG. 2 The $I-V$ and conductivity (dI/dV) curves of a cleaved surface of Bi₂CaSr₂Cu₂O₈.

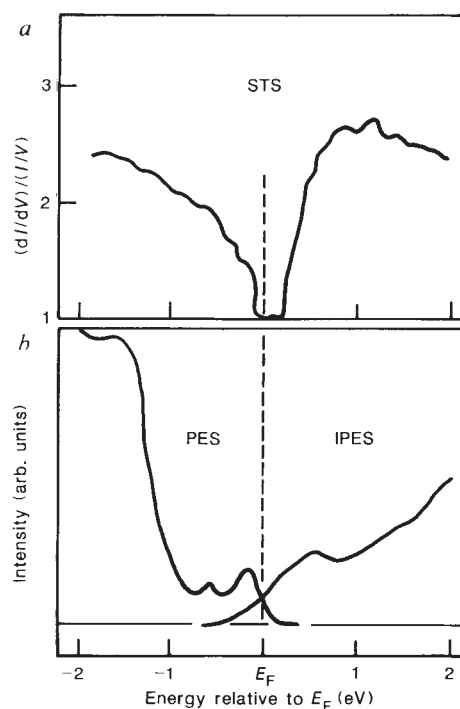


FIG. 3 *a*, STS spectrum of a cleaved surface of Bi₂CaSr₂Cu₂O₈. The normalized conductivity, $[(dI/dV)/(I/V)]$, is a measure of the surface density of states. *b*, Photoemission (PES)¹ and inverse photoemission (IPES)²⁰ spectra in the vicinity of the Fermi level (E_F).

tend to diverge within the gap region. They showed that this unphysical divergence can be avoided by choosing a large interval of bias voltage (ΔV) in the numerical calculation of the normalized conductivity curve. The curve in Fig. 3 shows no divergent behaviour although it exhibits a small gap at E_F . This suggests that the ΔV used in the present STS measurement is sufficient to give an accurate density of states even in the band-gap region. Thus, our STS results in Figs 2 and 3 show the existence of a small energy gap of about 0.3 eV in the surface density of states, implying that the BiO planes in bulk are non-metallic.

Also shown in Fig. 3 are the photoemission (PES)¹ and inverse photoemission (IPES)²⁰ spectra for comparison. These spectra show a clear Fermi edge and a substantial density of states at E_F , taking into account the resolution in each case (0.2 eV for PES and 0.4 eV for IPES). Given the difference in probing depth between PES (which probes a few atomic layers) and STS (which probes only the first layer), we conclude that the BiO plane is non-metallic and therefore that the Fermi-liquid states observed by PES and IPES are located in the CuO₂ planes. The existence of a small energy gap in the BiO planes contrasts with band-structure calculations⁶⁻⁸, which have predicted a continuous Bi 6p-O 2p band across E_F and thus a substantial density of states at E_F . This discrepancy may be due to the fact that the calculations did not use an accurate structural model: the small energy gap observed by STS could arise from the incommensurate superstructure of the BiO plane. A similar experimental result has been obtained recently by Shih *et al.*²¹ using STM/STS. Our conclusion is also supported by a recent study using

resonant electron Raman spectroscopy, which showed that lattice vibrations in the BiO plane do not couple with the holes in the Fermi level²².

Thus STS combined with PES and IPES reveals that the Fermi-liquid states in Bi₂CaSr₂Cu₂O₈ reside not in the BiO planes but in the CuO₂ planes. This description of the Fermi-liquid states should provide an experimental basis on which to build models of high- T_c superconductivity in the copper oxide materials. □

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Spin-glass models as error-correcting codes

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DURING the transmission of information, errors may occur because of the presence of noise, such as thermal noise in electronic signals or interference with other sources of radiation. One wants to recover the information with the minimum error possible. In theory this is possible by increasing the power of the emitter source. But as the cost is proportional to the energy fed into the channel, it costs less to code the message before sending it, thus including redundant 'coding' bits, and to decode at the end. Coding theory provides rigorous bounds on the cost-effectiveness of any code. The explicit codes proposed so far for practical applications do not saturate these bounds; that is, they do not achieve optimal cost-efficiency. Here we show that theoretical models of magnetically disordered materials (spin glasses) provide a new class of error-correction codes. Their cost performance can be calculated using the methods of statistical mechanics, and is found to be excellent. These models can, under certain circumstances, constitute the first known codes to saturate Shannon's well-known cost-performance bounds.

Spin-glass theory has already found applications to several fields outside its original scope, such as combinatorial optimization, associative memories, categorization theory and theoretical models of the immune system. Here I show that coding theory may be added to this list. Information theory, which forms the basis of coding theory, is also relevant to many of these other fields, and can in some sense be considered as their unifying element.

Information data are often represented in digital form, that is, as a sequence of symbols a_i equal to zero or one. Consider an electric signal v_i sent during a time interval τ . We set $v_i = v$

if $a_i = 1$ and $v_i = -v$ if $a_i = 0$. Let a_{ij} ($i, j = 1, \dots, m$) be the m^2 bits of information to be transmitted. Together with the a_{ij} s we send redundant bits of information ('parity' bits) defined through the redundancy relations $a_{i,m+1} = \sum_{j=1, \dots, m} a_{ij}$ and $a_{m+1,j} = \sum_{i=1, \dots, m} a_{ij}$, where the addition is modulo 2. We complete the square with $a_{m+1,m+1} = \sum_{i=1, \dots, m} a_{m+1,i}$. The ratio $R = m^2/(m+1)^2$ is called the rate of the code and measures the redundancy. The signal one receives is not the v_{ij} , but instead $u_{ij} = v_{ij} + y_{ij}$, where y_{ij} represents the noise. In an ideal case the y_{ij} are independent random variables with a zero-mean gaussian distribution. (This is called the gaussian channel.) From the sign of the u_{ij} one constructs the binary matrix b_{ij} . If the noise is extremely weak, then $b_{ij} = a_{ij}$ and all the rows and columns of the b_{ij} binary matrix will separately satisfy the redundancy relations. If these relations are not satisfied in the row i and column j , reversing the value of b_{ij} then corrects transmission errors, provided that there is only one error per row and per column. This simple error-correcting code belongs to the family of Hamming codes^{1,2} and works well for low noise levels.

Consider now the spin hamiltonian

$$-H = \sum_{i,j=1, \dots, m} u_{ij} \sigma_{ij} + \sum_{i=1, \dots, m} u_{i,m+1} \sigma_{i1} \sigma_{i2} \dots \sigma_{im} \\ + \sum_{i=1, \dots, m} u_{m+1,i} \sigma_{1i} \sigma_{2i} \dots \sigma_{mi}$$

The σ_{ij} are Ising spins, equal to ± 1 , the u_{ij} ($i, j = 1, \dots, m$) are external magnetic fields and the $u_{i,m+1}$ and $u_{m+1,i}$ are m -spin couplings. If one makes the correspondence $a_{ij} = (\sigma_{ij} + 1)/2$ between information bits and Ising spins, modulo 2 addition and spin multiplication are equivalent. If we take the u_{ij} s defined by the Hamming code and if σ_{ij}^0 denotes the spins corresponding to the ground state of H , the matrix elements $b_{ij} = (\sigma_{ij}^0 + 1)/2$ are the same as those that we get by decoding the Hamming code. To see this in the weak-noise limit, first forget the redundant bits $a_{i,m+1}$, $a_{m+1,i}$ and the m -spin couplings. The σ_{ij}^0 are aligned parallel to the magnetic fields u_{ij} . The redundancy relations are satisfied automatically and the m -spin couplings take their maximum value. This is the first and most trivial

example of equivalence between a spin model (in this case an m -spin-coupling version of the so-called random-field model) and an error-correction code.

More generally, the important parameters in information transmission are the energy per bit of the source E_s ($=\tau v^2$ in our example), the noise characteristics of the transmission channel (the variance w^2 in the case of the gaussian channel, the energy per bit of the noise being denoted as $N_0 = 2w^2$), the rate $R = (\text{number of information bits})/(\text{total number of transmitted bits})$ and the error probability per bit, P_e , after decoding. The performance of the code is measured by the error probability P_e as a function of the signal-to-noise energy ratio per bit of information, $S = E_s/(N_0 R)$. It can be shown that for given values of E_s/N_0 and R , P_e cannot be made arbitrarily small. Only certain regions in parameter space are allowed and it is remarkable that these regions are known¹. In the case of the gaussian channel there exists a code such that it is possible to communicate without any error if and only if $R \leq \frac{1}{2} \log_2 (1 + 2E_s/N_0)$. If $E_s/N_0 \ll 1$ this bound is $R \leq (1/\ln 2)E_s/N_0$. This is Shannon's famous result for the gaussian channel. No code saturating this bound is known. I will now show that there exists a family of codes that have a one-to-one correspondence with spin-glass models and that asymptotically saturate Shannon's bound.

Spin glasses² are disordered magnetic materials in which there is randomness in the position of individual magnetic moments. As their mutual interaction depends on their distance, there is also randomness in the interaction and they are usually modelled with the Edwards-Anderson hamiltonian

$$H_{EA} = - \sum_{i,j=1,N} C_{ij} J_{ij} \sigma_i \sigma_j$$

We first consider the case where the σ_i s are Ising spins. We call C_{ij} the connectivity matrix, the elements of which are 1 if the two spins interact and 0 otherwise. The J_{ij} s give the strength of the two-spin interaction and are usually taken as independent random variables with a known probability distribution. Let J_0 denote the mean and ΔJ^2 the variance of this distribution.

Two results of spin-glass theory will be of interest to us. First, at zero temperature (and for not too weak a connectivity) there is a phase transition: for $J_0/\Delta J$ greater than a certain critical value there is a spontaneous magnetization $m = 1/N \sum_i \sigma_i \neq 0$, whereas below this value $m = 0$ (here N is the number of spins). Second, the configuration space of the spins is invariant under the transformation $\sigma_i \rightarrow \sigma_i \varepsilon_i$ where the ε_i s are an arbitrary configuration of Ising spins. As $\varepsilon_i^2 = 1$ the hamiltonian is also invariant under this transformation and under the simultaneous transformation of the J_{ij} s, $J_{ij} \rightarrow J_{ij} \varepsilon_i \varepsilon_j$. This invariance property is called gauge invariance and can be shown to be identical to the gauge invariance of electromagnetism.

The above two properties are also true for the following hamiltonian, a straightforward generalization of the previous one:

$$H = - \sum_p \sum_{i_1, \dots, i_p=1, \dots, N} C_{i_1, \dots, i_p}^{(p)} J_{i_1, \dots, i_p} \sigma_{i_1} \dots \sigma_{i_p} \quad (1)$$

Let again $J_0^{(p)}$ and $\Delta J_{(p)}^2$ be the mean and the variance of the J_{ij} s.

I suggest now the following family of codes. Let a_i be the N bits of information and $\varepsilon_i = 2a_i - 1$ the spins associated with them. The coded message, that is, the input to the channel, corresponds to the matrix elements $J_{i_1, \dots, i_p}^0 = \varepsilon_{i_1} \dots \varepsilon_{i_p}$. Decoding corresponds to finding the ground state of the hamiltonian in equation (1). The J s in (1) are the output of the channel and are equal to the J^0 s plus noise. Any particular code in the family is specified by the connectivity matrices C . To estimate the performance it is enough to consider the case in which all the input bits are equal to unity. (The general case is the same because of the gauge symmetry.) In this case the number of errors is the number of spins that are equal to -1 , that is, the bit error probability is $P_e = (1 - m)/2$, where m is the ground-state magnetization.

I assume now for simplicity that there is only one value of p

in equation (1), and that the coordination number $z_i = \sum_{j_2, \dots, j_p} C_{i, j_2, \dots, j_p} = z$ is independent of i . Then the rate of the code is $R = p/z$. When the noise is very weak almost all of the σ s are equal to 1 and P_e can be computed. It is given by the probability that the sum of all the couplings to a spin be negative:

$$P_e = \int_{-\infty}^0 \frac{dy}{\sqrt{2\pi}} \exp\left(-\frac{y^2}{2}\right) \quad (2)$$

(The 'gain'^{1,2} of the code is $G = 10 \log_{10} p$ db, independent of R . This suggests it is advantageous to use large p .)

When the connectivity is extensive, that is, $z \sim \binom{N-1}{p-1}$, it has been shown³ that only the two first moments of the noise distribution are relevant, and one has to rescale the couplings, $J_0 = (p!/N^{p-1})j_0$, and $\Delta J^2 = (p!/N^{p-1})\Delta j^2$, to get a sensible thermodynamics. We set $\Delta j = 1$ and $\tau = 1$ to fix the normalization. If $p = 2$ or $p \rightarrow \infty$, and $p/N \rightarrow 0$, the spin-glass model is soluble. For $p \rightarrow \infty$ it is Derrida's random-energy model (REM)⁴. It can be shown that for $j_0 > j_{0,c}$ the spontaneous magnetization $m = 1$, whereas for $j_0 < j_{0,c}$, $m = 0$, where $j_{0,c} = \sqrt{\ln 2}$. Using the replica method for large p (refs 5, 6) one can also show that $j_{0,c}$ decreases with p , and for $j_0 \approx \sqrt{\ln 2}$

$$m = 1 - \frac{\exp(-pj^2)}{\sqrt{p}} c(j) \quad c(\sqrt{\ln 2}) = 0.987 \quad (3)$$

Thus the approach to asymptotia is very fast. (The details of the computations will be published elsewhere.)

The $p = 2$ case is the Sherrington-Kirkpatrick model⁷. This model develops a spontaneous magnetization (m_{SK}) for $j_0 > j_{0,c} = \frac{1}{2}$ (our normalization for j_0 differs by a factor of 2 from the usual one) which has been computed analytically⁸ and numerically⁷. For $j_0 = \sqrt{\ln 2}$, $m_{SK} = 0.85$.

So the REM is, at least in theory, an ideal error-correction code; for $j_0 > \sqrt{\ln 2}$ one can recover the input without error and the approach to asymptotia is as given by equation (3). By solving the REM we obtain Shannon's result by an entirely novel route. (This involves using the relations $v^2/w^2 = j_0^2 p!/N^{p-1}$ and $R = p!/N^{p-1}$.)

No exact algorithm is known for minimizing H and one has to use heuristic methods such as simulated annealing^{9,10} (essentially a computer simulation of the thermodynamics of the model) which provides only an approximation to the ground state.

I have performed preliminary numerical simulations for $p = 2$ and $R = \frac{1}{3}$ and $R = \frac{1}{4}$. Starting from random initial conditions,

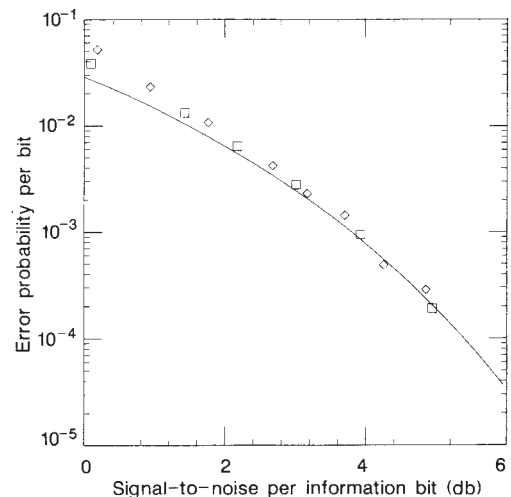


FIG. 1 Error probability per bit for different signal-to-noise power ratios. The solid line represents the analytic solution of the SK model, squares are simulations for $R = \frac{1}{3}$ and diamonds for $R = \frac{1}{4}$ spin-glass codes with $p = 2$.

the Monte-Carlo algorithm was run using 100 steps per spin, decreasing the temperature gradually to zero. The results are shown in Fig. 1. The performance is independent of R down to 1 db, as suggested by equation (2), and is independent of N . Because the Monte-Carlo algorithm can be made parallel, custom circuits of the neural-network type can be used for decoding. Neural-network models for error-correction codes have been considered by Platt and Hopfield¹¹.

The simulations show that simulated annealing works very well as a decoding algorithm; one may wonder why this is, because it is known that finding the ground state of spin glasses is an N -complete optimization problem. The simple reason is that we are interested here in the ferromagnetic phase and not in the spin-glass phase. Furthermore, at the noise levels that concern us here (down to ~ 1 -0 db per information bit) it can be shown that the error does not depend on the exact determination of the ground state ('replica symmetry breaking'³, the appearance of quasidegenerate metastable states, occurs at very low temperature and the magnetization does not vary below that temperature).

Clearly the central issue for practical applications is the performance of approximate decoding and a more systematic numerical study is needed, but these first results are very encouraging.

In addition to the Hamming codes, many other common codes can be written as spin models, because of the mathematical equivalence between modulo 2 addition and spin multiplication. I shall illustrate this with the $R = \frac{1}{2}$ Viterbi code, for which the equivalence with spin models has first been noticed by J. Hopfield (personal communication). The input sequence can be formally represented as a polynomial $P(x) = \sum_{i=0,1,\dots} a_i x^i$, and the coded input to the transmission channel consists of the two polynomials $G_j(x) = g_j(x)P(x)$, $j = 1, 2$. The $g_j(x)$ are called the generator polynomials of the code. Choosing $g_1 = 1 + x^2$ and $g_2 = 1 + x + x^2$, $G_1(x) = \sum_i (a_i + a_{i-2})x^i$ and $G_2(x) = \sum_i (a_i + a_{i-1} + a_{i-2})x^i$, where addition of the a_i s is modulo 2. Decoding is equivalent to finding the ground state of $H = -\sum_i (X_i^{(1)} \sigma_i \sigma_{i-2} + X_i^{(2)} \sigma_i \sigma_{i-1} \sigma_{i-2})$, where $X_i^{(j)}$ are the output noisy G_j coefficients. This is a one-dimensional spin-glass model with short-range interactions. Viterbi decoding is a deterministic algorithm and is widely used. Equation (3) suggests that better performance could be achieved by using higher-degree generating polynomials, with longer-range interactions, and by decoding approximately through simulated annealing. (The deterministic algorithms become intractable as the degree of the generating polynomial increases.) This possibility has to be investigated more systematically by numerical techniques.

Instead of Ising spins, it is also possible to consider q -state clock-model spin glasses. This means using modulo q instead of modulo 2 arithmetic. It is possible to consider even more general systems with vector spins and non-abelian discrete symmetry groups (the crystallographic groups). These kinds of code are now under investigation. $\square$

Does the gas content of amber reveal the composition of palaeoatmospheres?

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WHAT does the gas content of amber indicate? It has been suggested that bubbles in amber preserve the composition of the atmosphere at the time the amber was exuded from trees^{1,2}, while others suggest that the gas content is controlled by solubility relationships³, diffusion⁴ or chemical reactions involving oxygen consumption⁵. Here I describe a combined study of nitrogen, argon and oxygen in amber which makes it possible to distinguish bubble gas from matrix gas in amber by considering N_2/Ar and O_2/Ar ratios of the first gas released during the crushing of individual amber samples. Crushing experiments do not indicate high oxygen levels in Cretaceous amber as have been recently reported¹. Neither Baltic nor Cretaceous amber has even the amount of oxygen expected for equilibration with the modern atmosphere. Successive crushings of individual amber pieces show that the first crush has the highest oxygen content and that both bubble and matrix oxygen is consumed as crushing continues. These results suggest that the oxygen content of amber does not have a bearing on the composition of the palaeoatmosphere.

Argon is an inert gas, nitrogen is fairly unreactive and both gases have long residence times in the atmosphere⁶. In contrast, oxygen has a much shorter residence time in the atmosphere and is more reactive with organic compounds. The nitrogen/argon ratio (N_2/Ar) in the atmosphere should therefore have been constant over the past 100 Myr. Nitrogen, argon and oxygen have very different solubilities in fluids. In water, the solubility of nitrogen is $\sim 47\%$ that of argon, whereas oxygen is only 9% less than argon. Because argon is an inert gas and has a solubility similar to that of oxygen, argon serves as a useful tracer of the physical processes that affect oxygen⁷. The N_2/Ar ratio is therefore considered to be a measure of the matrix (solubility) component or atmospheric (bubble) component of gas, whereas the O_2/Ar ratio is considered to be a measure of the relative reactivity or the excess nature of oxygen in amber.

Sequential crushings of amber were carried out in a miniature crusher which was attached on-line to a quadrupole mass spectrometer. The volume of the crusher was 1.9 cm^3 and could be separated from the inlet portion of the line which had a volume of 9 cm^3 . Gases were allowed through a -195°C trap and analysed dynamically on the quadrupole mass spectrometer. This trapping temperature removed hydrocarbons which interfered with Ar measurements in early experiments using higher trapping temperatures; it also removed CO_2 which was not therefore analysed.

Previously, Horibe and Craig³ found that the N_2/Ar ratio for dissolved gases in amber and modern tree resin is between 37 and 39, which is remarkably similar to that in water, 38.4 at 22°C . Thus, it seems that the ratios for the solubility of gases in amber and tree resins are similar to that in water. By analogy, it is expected that the solubility ratio for O_2/Ar in amber in equilibrium with the atmosphere would also be similar to that in water. It is convenient to present the analytical results as R/R_a ratios of N_2/Ar and O_2/Ar relative to the atmosphere where R is the ratio in the sample and R_a is the ratio in the atmosphere:

$$(N_2/Ar)R/R_a = (N_2/Ar)_{\text{sample}}/(N_2/Ar)_{\text{atmosphere}}$$

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The equilibrium solubility R/R_a values for gases dissolved in the matrix of amber are therefore considered to be 0.47 and 0.91 for N_2/Ar and O_2/Ar , respectively.

To establish that bubbles in amber have the atmospheric N_2/Ar ratio, bone amber was analysed by the sequential crushing technique. Bone amber is opaque owing to numerous small bubbles which are $\sim 1\text{--}10\ \mu\text{m}$ in diameter. It is only with bone amber that one can be sure that a random crush intersects many bubbles. Bone amber is likely to exchange with the modern atmosphere⁴ because of the short path length from the atmosphere to the bubbles and so it also provides a means of testing for the reactivity of uncrushed amber with oxygen from the modern atmosphere.

Figure 1 shows that successive crushings of bone amber have constant (N_2/Ar) R/R_a ratios which are close to the modern atmospheric values and indicate that the crushing is releasing only bubble gas. However, the (O_2/Ar) R/R_a ratio decreases as the later gases are released indicating that oxygen in the bubbles is reacting with the amber after crushing begins. The first crush gave an (O_2/Ar) R/R_a ratio of only ~ 0.24 that of the atmosphere indicating that oxygen in bubbles in uncrushed bone amber has not yet equilibrated with the atmosphere. The low R/R_a ratio for the initial O_2/Ar implies that oxygen is being consumed by amber in its uncrushed state. The decrease in (O_2/Ar) R/R_a ratio with time suggests a mean life of $\sim 1,040$ s for oxygen in Baltic bone amber under these crushing conditions. The oxygen in crushed amber is probably consumed by free radicals that are formed by mechanical grinding of amber⁸. This sequential crushing technique also allows extrapolation back to the start of crushing, which gives virtually the same O_2/Ar value as measured for the gas released by the first crush. Such an extrapolation would minimize problems of back reaction with amber and adsorption onto the walls of the extraction line⁹.

The sequential crushing method was used to analyse four Cretaceous amber samples from Mt Hermon, Israel¹¹, and Cedar Lake, Manitoba², and four Tertiary Baltic amber samples. Figure 2 shows that the first gas released from each amber sample had a N_2/Ar ratio very close to a bubble or matrix component. However, the first gas released for all samples was deficient in O_2 , having an (O_2/Ar) R/R_a ratio < 0.6 . Cretaceous amber was found to have a higher O_2 content than Baltic amber—in line with results from other workers^{1,9}—but these results do not suggest O_2 levels higher than today. As the first gas released has lower-than-modern O_2/Ar ratios, this suggests that oxygen is reacting with amber. Continued crushing of these samples showed that oxygen was gradually consumed, having a mean life of between 1,000 and 4,000 s in crushed amber. Most Cretaceous amber samples have N_2/Ar ratios that are indicative

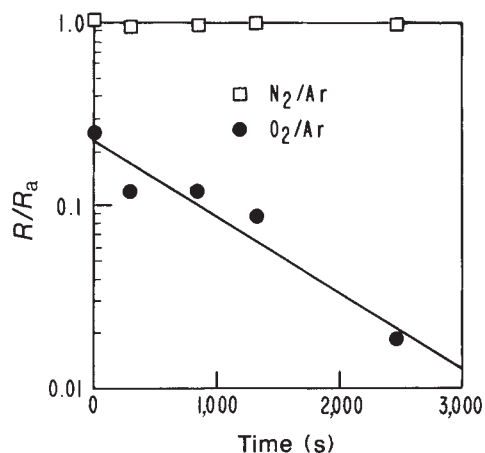


FIG. 1 R/R_a values for gases released during sequential crushing of Baltic bone amber plotted against time after the initial crush.

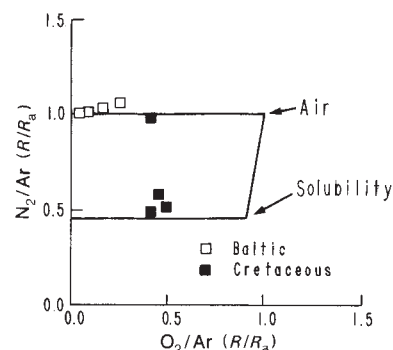


FIG. 2 R/R_a values for N_2/Ar and O_2/Ar for Baltic amber and Cretaceous amber. The upper horizontal line represents a mixing line between the atmosphere and an atmosphere with no oxygen; the lower horizontal line represents a mixing line between the solubility ratio and the solubility ratio with no oxygen; the atmospheric value and solubility value are also connected by a mixing line. R/R_a ratios lying within these lines can be explained by mixing of bubble and matrix gas and oxygen consumption reactions. Only values falling outside of the box could be construed as providing evidence of excess oxygen. All points are for the first gas released by crushing.

of matrix gas rather than bubble gas because they fall very near the equilibrium solubility value. These samples have O_2/N_2 ratios that are similar to the atmospheric value but result from the higher solubility of O_2 relative to N_2 in the matrix.

Measurements of the inert gases N_2 and Ar in amber therefore show that inert gases in bubbles and in matrix are in equilibrium with the atmosphere. The initial O_2/Ar ratio in Baltic bone amber, however, suggests that oxygen reacts with amber. Based on the relatively high diffusion coefficients measured for gases in amber⁴, and the observation that museum specimens almost invariably become darker with time^{5,10}, it is likely that modern atmospheric gases freely exchange and react with amber on exposure to the atmosphere. Most amber has been exposed to atmospheric oxygen levels only relatively recently, and therefore has a cumulative exposure time to atmospheric oxygen measured in decades. Crushing promotes reaction of amber with oxygen resulting in more rapid oxygen consumption than in the uncrushed state. By considering both the N_2/Ar and O_2/Ar ratios it is possible to determine if the gas being analysed was derived from bubbles or from the matrix and therefore it is not necessary to assume that the gas being analysed was derived from bubbles¹. The N_2/Ar and O_2/Ar ratios of gases released from Cretaceous amber indicate an O_2 level that is lower than atmospheric, not higher as has been previously suggested^{1,9}. The low O_2/Ar ratios probably result from oxygen consumption by amber in the uncrushed state which means that the oxygen content of amber does not reflect the composition of the palaeoatmosphere. □

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Melting in an Archaean mantle plume: heads it's basalts, tails it's komatiites

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THE lower part of most Archaean greenstone sequences is dominated by interlayered basaltic and komatiitic (ultrabasic) flows. These two magma types are petrologically and geochemically distinct, yet they display a close spatial and temporal association. The origin of the anomalously high-temperature komatiitic liquids has been much debated because of the implications for the thermal structure and composition of the Archaean mantle. Here we argue that both the basalts and komatiites are produced by a starting thermal plume rising in a warmer Archaean mantle. Fluid-dynamics studies show that a starting plume consists of a hot axial jet, capped by a large head into which cooler surrounding mantle is entrained. Our calculations for such a flow indicate that komatiites could form by melting in the high-temperature axis of the plume and basalts by melting in the cooler head. The sudden onset and limited duration of the basalt/komatiite sequences, seen in the greenstone belts of Western Australia and elsewhere, are explained by this model.

Major greenstone sequences, such as the Abitibi in Canada, the Barberton in South Africa and the Norseman-Wiluna in Western Australia, consist of a lower series made up of basalts and komatiites overlain by felsic volcanics and sediments. The Kambalda section of the Norseman-Wiluna greenstone belt is typical of the lower greenstones. It consists of a thick footwall sequence of tholeiitic basalts overlain by komatiites which in turn are overlain by the thinner hangingwall basalts. The latter show clear evidence of crustal contamination¹⁻³ and are therefore of limited value in drawing conclusions about their mantle source region. The komatiites and footwall basalts, however, show continuous light-rare-earth-element (LREE) depletion through to La and are apparently free from this problem. Furthermore, they are unlikely to be related through fractional crystallization³. The komatiites overlie the footwall basalts, which is the reverse of what is expected if the basalts were derived from the komatiites. The komatiites also have distinctly higher ϵ_{Nd} (see Fig. 1 legend) and lower chondrite-normalized Nd:Sm ratio, $(\text{Nd}/\text{Sm})_{\text{c}}$, than the basalts (Fig. 1), ruling out a common mantle source.

The maximum MgO content of the Kambalda footwall basalts is 14 wt%, as opposed to 32 wt% for the komatiites^{4,5}, implying a difference in liquidus temperature of 350 °C (that is, 1,650 °C as opposed to 1,300 °C)^{6,7}. This temperature difference can be used to estimate the temperature difference between the mantle sources for the two magmas, provided that differences in the degree of partial melting and in the adiabatic temperature paths are taken into account^{8,9,13}. If it is assumed that 20% more melting is required to produce a komatiite, this correction is ~100 °C. We therefore estimate the temperature difference between the mantle sources for Archaean tholeiites and komatiites to be 450 °C. The uncertainties in this calculation are, however, considerable, and could be as high as 100 °C. The corresponding potential temperatures (T_{p}), the temperatures of the mantle sources for the tholeiites and komatiites at a pressure of 1 bar (ref. 8), are approximately 1,425 °C and 1,875 °C, respectively, compared with $T_{\text{p}}=1,280$ °C and up to 1,530 °C for mid-ocean-ridge basalts (MORBs) and hotspot basalts, respectively⁸. The higher the temperature of the mantle, the greater the pressure at which melting can begin. Basalts and komatiites may thus be formed by melting different sections of the mantle at different temperatures and pressures.

If the source of Archaean komatiites had a potential temperature almost 600 °C above that of the modern mantle (away from hotspots) there are two possibilities: the Archaean upper mantle was 600 °C hotter than the modern mantle¹⁰, or komatiites came from anomalously hot zones within the mantle¹¹⁻¹³. At $T_{\text{p}}=1,875$ °C, the top of the upper mantle, with the exception of a thin solid skin, would be completely molten, and a high degree of partial melting would extend to a depth of several hundred kilometres. This would lead to ancient geothermal gradients larger than their modern equivalents, a prediction that is inconsistent with gradients determined from Archaean metamorphic assemblages^{14,15}. Furthermore, geochemical arguments, based on the strong partitioning of incompatible elements into minerals with the perovskite structure, indicate that the modern mantle would not have its observed near-chondritic abundance of these elements if a significant volume of the early mantle has been molten^{16,17}. We therefore conclude that anomalous temperatures are required for the formation of Mg-rich Archaean komatiites. In a convecting mantle these temperatures will be found in a hot upwelling plume.

In elaborating the plume hypothesis, we note that there appears to have been a sudden and widespread onset of activity in the Norseman-Wiluna belt 2.7 Gyr ago, after a long quiescent period, and that most of the basalts were erupted during a period of only 15 Myr (ref. 21). Laboratory and theoretical studies^{18,19} show that a new plume, originating from a boundary layer deep within the mantle, cannot displace the overlying mantle and ascend rapidly until a large amount of buoyancy has collected in a nearly spherical 'head'. A narrow conduit, or 'tail', can form in the path of the head and allows rapid ascent of hot, low-viscosity material. If there is a sufficient supply of hot material, the head can be constantly fed by the source for some time and can continue to grow in size as it ascends. Fluid-dynamics studies show also that entrainment of surrounding mantle into the plume head is inevitable^{19,20} (Fig. 2). The head therefore ultimately contains material both from the hot source region and from the cooler mantle through which the plume head passes. As a result, the temperature in the bulk of the head

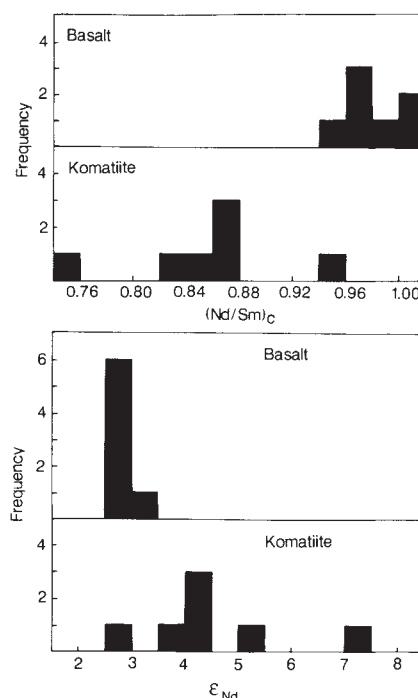
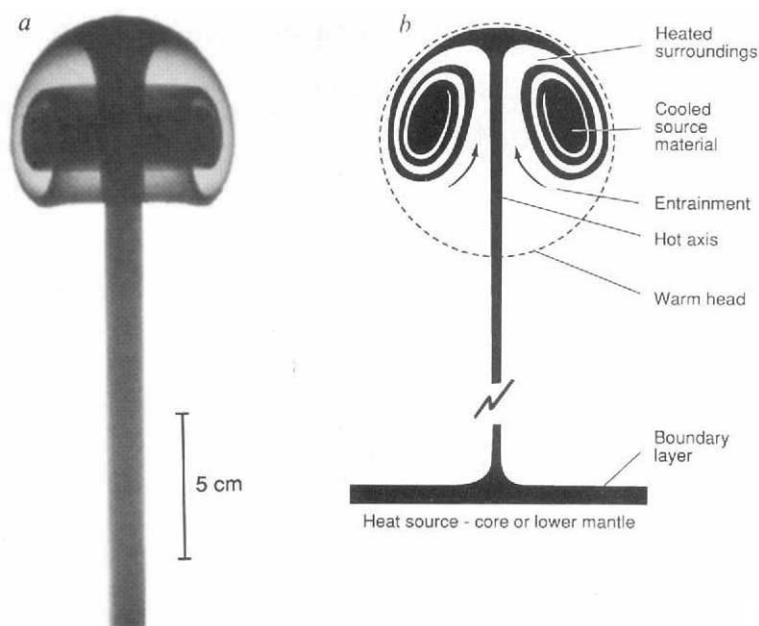


FIG. 1 Variations in the chondrite-normalized Nd:Sm ratio, $(\text{Nd}/\text{Sm})_{\text{c}}$, and in ϵ_{Nd} for basalts and komatiites from Kambalda. Data from refs 2, 28, 29. $\epsilon_{\text{Nd}} = ((I_{\text{S}}/I_{\text{CHUR}}) - 1) \times 10^4$ where I is the $^{143}\text{Nd}/^{144}\text{Nd}$ ratio, S refers to the sample and BE refers to the bulk Earth.

FIG. 2 *a*, A photograph of a starting thermal plume generated in a laboratory tank of very viscous glucose syrup (Reynolds number $Re \approx 2 \times 10^{-3}$). The fluid temperature was 21 °C (viscosity = 130 st). Some of the same syrup was coloured with dye, heated to 80 °C (viscosity ≈ 0.1 st) and injected at the base at a constant flow rate of $0.3 \text{ cm}^3 \text{ s}^{-1}$. The distribution of heat cannot be seen here, but shadow-graph images of the flow are consistent with a spherical head outlined by a sharp temperature front. *b*, Schematic sketch (based on laboratory observations) of a starting thermal plume before it begins to spread near the top of the mantle and before significant melting. The black material comes from the plume source region and may be chemically and isotopically different from the surrounding mantle. Conduction of heat during the rise of the plume causes entrainment of cooler mantle and smoothing of temperature gradients within the head, where the mean temperature is between those of the source and ambient mantle. The head can be modelled as a spherical volume of uniform temperature²⁰, apart from the axial conduit. If both materials in the head partially melt, the migrating melts will mix. Melts from the plume axis, or tail, will be hotter and relatively uncontaminated.



is appreciably less than that along the axis (from the tail to the leading edge of the plume), where the potential temperature of the source prevails. This plume structure is consistent with that found in many numerical simulations of high-Rayleigh-number convection above a heated lower boundary (see, for example, refs 12, 21). Mathematical modelling, based on a similarity solution for an isolated diapir^{19,20}, but adding a constant flux from the source region, indicates that the required temperature difference of 450 °C between head and tail can be achieved in plumes rising from the core-mantle boundary under conditions appropriate to the Archaean mantle (Fig. 3). The longevity of flow in the plume tail cannot be predicted at this stage, but is likely to be shorter in a hotter, more unsteady Archaean mantle than it is today.

The rise of a starting plume has been proposed as an explanation for widespread mantle and crustal anatexis, which resulted in the large out-pouring of basalt and associated granitoid intrusion that extended over much of the Yilgarn Block 2.7 Gyr ago²². Starting plumes also provide an explanation for the formation of more recent flood basalts, which appear to lie at the beginning of linear hotspot tracks^{23,24}. We now suggest that the Archaean komatiites were formed by melting in the high-temperature axis of a starting plume. We note that picrites, rather than komatiites, are found associated with more recent flood basalts, probably because the modern mantle is cooler.

It has been suggested¹¹⁻¹³ that komatiites are derived from melting in an established plume or, more specifically¹³, where there is a coincidence between lithospheric stretching and mantle 'jets'. The starting-plume model now offers an explanation for this coincidence: the plume head will uplift the mantle lithosphere and continental crust by about 2 km (refs 25, 26), causing lithospheric stretching and the tensional features that formed during the early stages of greenstone development²⁷. The model predicts many other characteristics of the development of granite-greenstone terrains: the association between high-temperature komatiites and the more voluminous lower-temperature tholeiitic basalts; the different source isotopic compositions at Kambalda (Fig. 1); the timescale for the eruption of basalts and komatiites (~ 15 Myr)²²; and the horizontal length-scale of the 2.7-Gyr crustal anatexis event ($1,000 \text{ km}$)²². The apparently short time interval for komatiite eruption suggests that either the large crustal tensions produced by the initial rise of the plume head were necessary for the rise of komatiite through the crust, or the flow up the plume tail did not continue for long¹⁹.

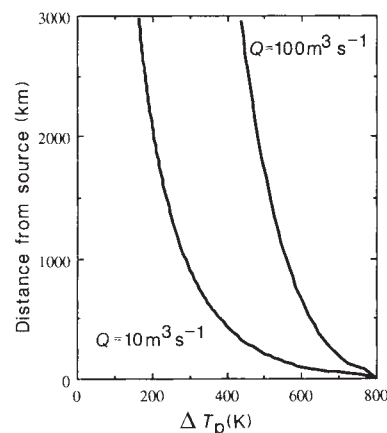


FIG. 3 The difference between the potential temperature in the head of a starting plume and that in the surrounding mantle, as a function of the height of rise of the head, for two source volume fluxes Q . The calculation is an extension of Griffiths's similarity solution for viscous thermals²⁰, and assumes no cooling along the plume tail. In this run the source (and hence the axial conduit of the plume) is assumed to be 800 °C hotter³⁰ than the surroundings. The flow rates used are comparable with those estimated for present-day hotspots. There is, however, considerable uncertainty in the choice of initial conditions (Q and ΔT_p) and the results of the calculations are presented solely to illustrate a principle. Note that the head cools rapidly as a result of entrainment. The results are independent of the mantle viscosity.

If our hypothesis is correct and the axis and tail of the plume contain uncontaminated material originating from deep in the mantle, some simple conclusions can be drawn about changes in the geochemistry of hotspot sources through time. The trace-element and isotopic characteristics of komatiites from Kambalda (Fig. 1) and elsewhere require the deep Archaean mantle source to have been depleted in LREEs for an appreciable period; it must have been similar to the modern MORB source. In contrast, modern plumes or hotspots are isotopically and geochemically enriched. Either these plumes have different sources, or the geochemistry of the hotspot source has changed from depleted in the Archaean mantle to enriched in the modern mantle. The latter possibility is supported by the scarcity of mantle-derived alkali basalts in the preserved Archaean crust. □

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Patterns in tree species richness as a test of the glacial extinction hypothesis

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It is well established that Europe has far fewer tree species and genera than either eastern North America or eastern Asia^{1–3}. Fossil evidence shows that west-central Europe had a much richer tree flora during the Upper Tertiary (25–2 Myr BP), with many genera which now survive only in temperate regions of North America and Asia³. These trees seem to have been eliminated from Europe during cold, dry glacial periods of the Pleistocene Era (2–0.001 Myr BP). Their failure to recolonize may be due to a failure of species to reach potential refugia during the glacial phase^{2,3}. Alternatively, present-day climates, rather than purely historical effects of extinction or survival in refugia, may explain the observed contrasts in richness between regions. Currie and Paquin⁴ showed that the number of tree species in Britain and Ireland corresponds to that of North American areas of similar climate, without the need to invoke the historical explanation. Here we report that the differences in species richness between three northern temperate

regions, Europe, eastern North America and eastern Asia, can be mainly explained in terms of present-day climatic factors. Similarly, and despite its long period of isolation from the northern temperate flora, the tree flora of New Zealand corresponds closely to the same relationship between climate and species richness. By contrast, greater species richness (compared with Europe and North America) found in some parts of eastern Asia cannot readily be accounted for in terms of present climate, and may be due to lower rates of extinction during glacial phases.

Our conclusions are based on an extensive study of climate-richness relationships for the whole of Europe and parts of eastern Asia (Soviet East Asia, Japan and Taiwan). We used natural range maps and local floras^{2,5–10} to produce totals of numbers of species of trees (defined as any woody species reaching 3 m or more in any part of its range) on a grid of large-scale quadrats (Table 1). Tree species richness per quadrat was regressed against topographic variability¹¹ and several climatic factors¹², including a model for predicting productivity of vegetation—the Chickugo Model^{13,14} (Table 1). The Chickugo Model was found to be the strongest predictor of variation in richness within Europe, eastern Asia and also (using Currie and Paquin's quadrat data, Currie, personal communication) in North America. The relationship between richness and predicted productivity is even stronger in the more mesic regions of North America and Europe (Table 1). The basis for this very close relationship is not clear, although various ecological and evolutionary explanations might be put forward^{1,15}.

As for North America⁴, most of the variation in tree species richness within Europe and Asia can be accounted for in terms of a present-day climatic index, without recourse to historical explanations. When scatterplots of richness against predicted productivity for different regions are superimposed (Fig. 1), it

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TABLE 1 Linear correlation coefficients

	Europe (166 d.f.)	North America (193 d.f.)	East Asia (106 d.f.)
Predicted NPP (Chickugo model)	0.915**	0.953**	0.915**
Latitude	−0.898**	—	−0.879**
Net solar radiation	0.883**	0.945**	0.890**
July mean temperature	0.779**	—	0.738**
January mean temperature	0.707**	—	0.668**
Mean annual temperature	0.850**	—	0.831**
Annual temperature range	−0.439**	—	−0.276**
Topographic range	0.588**	—	0.238*
Radiative dryness index	−0.386**	−0.562**	−0.057 n.s.

Linear correlation coefficients for mesic regions of the northern temperate zone; tree species richness per quadrat against various environmental factors. The quadrat sizes were 2.5 degrees × 2.5 degrees below 45 degrees N, 3.5 × 2.5 degrees from 45 to 60 degrees N and 5.0 × 2.5 degrees above 60 degrees N. These correspond to a mean quadrat area of ~72,000 km², ranging from 81,000 km² to 66,000 km². Within each cartesian size category, absolute quadrat size decreases towards the north. Climatic data values corresponding to the central point in each quadrat were obtained by interpolation. Climatic factors similar to those already analysed by Currie and Paquin⁴ are not included in this analysis. Predicted primary productivity values were calculated from global net solar radiation maps¹⁴ and annual precipitation, using the Chickugo Model of Uchijima and Seino¹³. The productivity value for each quadrat corresponds to the highest value for an area below 500-m altitude and occupying at least 5 per cent of the quadrat area. The radiative dryness index, which also incorporates solar radiation and precipitation data¹³, is used as an estimate of aridity. Note that the size of the correlation coefficient does not express the strongly non-linear component observed in many of the relationships. NPP, predicted net primary productivity. Significance level: *, $P < 0.05$; ** < 0.01 .

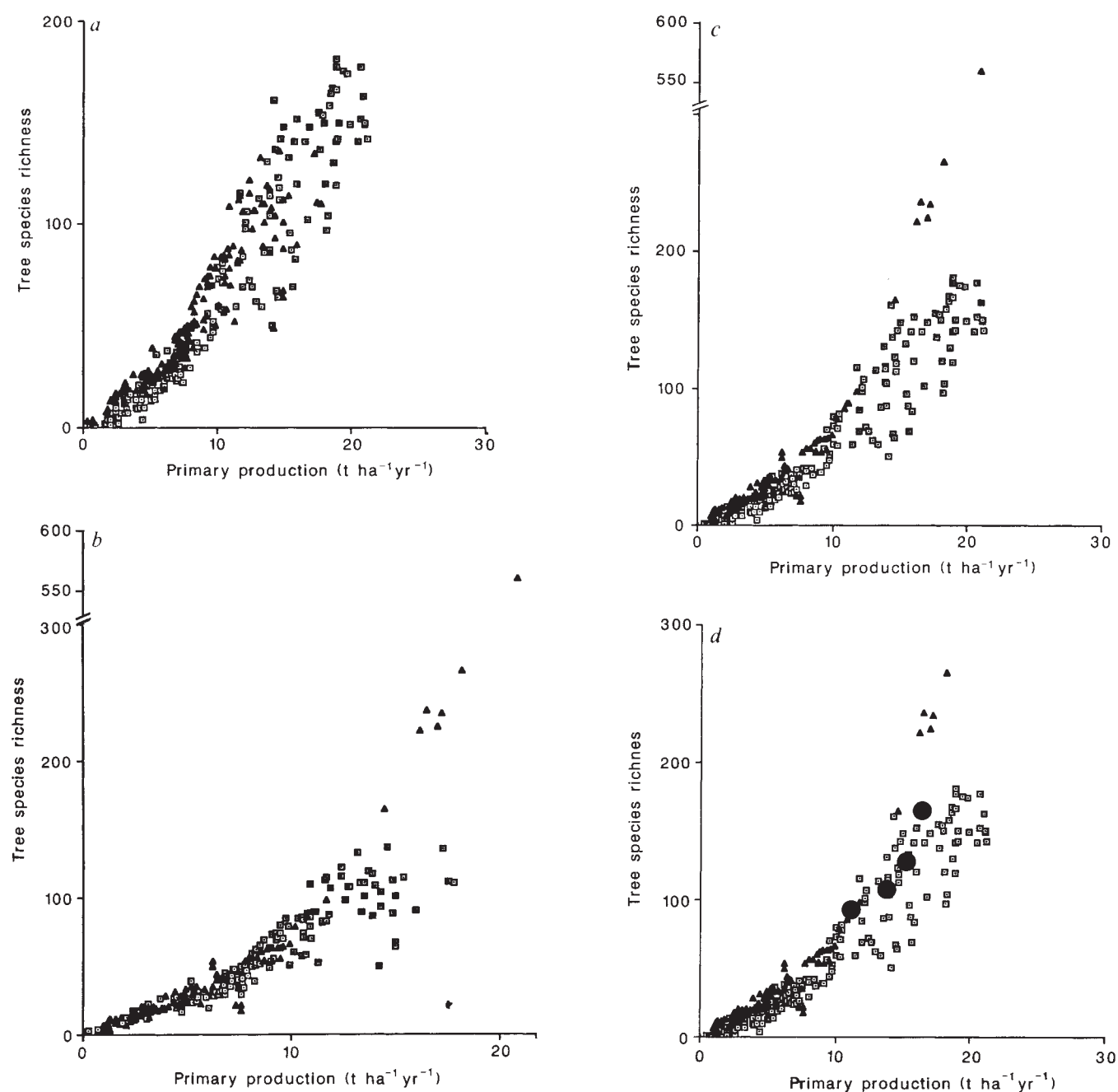


FIG. 1 Superimposed richness versus productivity scatterplots for the mesic areas (radiative dryness index¹³ <1.5) of three temperate regions of the Northern Hemisphere. Vertical axes; numbers of tree species per quadrat unit. Horizontal axes; annual primary productivity of natural vegetation

predicted using Chickugo model. *a*, Europe (triangles) and America (squares). *b*, Europe (squares) and East Asia (triangles). *c*, America (squares) and East Asia (triangles). *d*, North America (squares) and East Asia (triangles) with points from New Zealand (circles).

TABLE 2 Single-factor linear and non-linear regression analyses

Region	Variance explained (%)	Equation of best-fit line
Europe (total)	84.3	$\text{SPPS} = 6.75 + 109 / (1 - \exp(-0.37 \times (\text{NPP} - 8.97)))$
Europe (mesic)	87.4	$\text{SPPS} = 10.86 + 97.87 / (1 - \exp(-0.552 \times (\text{NPP} - 8.84)))$
East Asia	97.5	$\text{SPPS} = 5.21 + 747 \times 1.07^{\text{NPP}}$
North America (total)	80.8	$\text{SPPS} = -31.6 + 43.4 \times 1.07^{\text{NPP}}$
North America (mesic)	92.0	$\text{SPPS} = -10.7 + 192.8 / (1 + \exp(-0.232 \times (\text{NPP} - 12.4)))$

Results of single-factor linear and non-linear regression of tree species per quadrat (SPPS) versus predicted net primary productivity (NPP). Regression was carried out using the GENSTAT5 statistical package. In each case, the equation for the best-fit curve is given. For the mesic North American region, an exponential curve gave an r -squared value 2 per cent below that of a sigmoid curve. In all other cases the fitted curve gave an r -squared value 4.5 per cent or more above any other curve type. The very high r -squared value obtained for the East Asian region can be ascribed partly to the high leverage exerted by the single outlying data point for Taiwan.

becomes possible to test for historical effects acting between regions, which might have altered the productivity-richness relationship.

All three temperate regions show very similar trends in richness trends up to a predicted productivity of $\sim 15 \text{ t ha}^{-1} \text{ y}^{-1}$. At higher productivity levels, richness in Europe and North America continues to follow essentially the same trend, whereas in eastern Asia much higher levels of richness are reached at corresponding productivity levels.

These results challenge some widely held views on the role of glacial extinctions in producing differences in richness between the temperate regions. The tree flora of western and central Europe, which is often contrasted against the much richer floras of Japan, Taiwan and the south-eastern U.S.A., is in fact no less rich than areas of corresponding productivity levels ($7\text{--}13 \text{ t ha}^{-1} \text{ y}^{-1}$) in North America and eastern Asia. This is also found to be true at all other levels of productivity when Europe and North America are compared. Certain areas of the North American region do have higher species richness than Europe, but this can be explained simply in terms of the greater productivity reached in North America.

There may, however, be some glacial legacy in the contrast between the most productive areas of Europe and North America, on the one hand, and eastern Asia on the other. The trend of increasing richness with productivity may have been 'truncated' by extinction in Europe and America, to give sigmoid curves rather than the steeply rising exponential curve for eastern Asia (Table 2). Why should such an extinction effect have operated most strongly in high-productivity regions? Possibly, the trees which inhabit such environments, being more cold- and drought-sensitive, would be much less likely to 'find' suitable refugia than trees from less productive areas; hence they would be more likely to go extinct.

Despite its long history of separation (and very different taxonomic composition) parts of the temperate flora of the Southern Hemisphere show a very similar productivity-richness relationship to the floras of the Northern Hemisphere discussed above. Data points from the tree flora of New Zealand^{16,17} (Fig. 1, *d*) lie very close to the richness values of eastern Asia and North America. Such striking convergence implies some ecological mechanism acting in common, which tends to regulate the number of tree species present in an area. This appears to have overridden the effects of tens of millions of years of isolation and evolutionary divergence. □

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Expression of dopaminergic phenotypes in the mouse olfactory bulb induced by the calcitonin gene-related peptide

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IN the olfactory bulb, tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, is expressed after birth when the axons of olfactory epithelial neurons have made synapses in the bulb^{1,2}. It has been suggested that expression of TH is regulated trans-synaptically because on deafferentation of the bulb there is a marked decrease in the contents of TH, dopamine and 3,4-dihydroxyphenylacetic acid, which, however, return to normal levels after regeneration of the primary afferents^{3–6}. To date the molecular signalling involved in this trans-synaptic induction has not yet been characterized; I have therefore studied the expression of dopaminergic properties (presence of TH and dopamine uptake) in dissociated cell cultures from embryonic mouse olfactory bulb. I report that the number of dopaminergic cells increases fivefold when olfactory bulb neurons are co-cultured with olfactory epithelial neurons and that soluble factors, rather than cell interactions, mediate this effect. The dopaminergic-inducing factor is the calcitonin gene-related peptide (CGRP)^{7,8} which is present in chemosensory neurons of the olfactory epithelium and when added at nanomolar concentrations to olfactory bulb cultures mimics the effect of olfactory epithelial neurons. Significantly the induction of dopaminergic phenotypes brought about by olfactory epithelial neurons is abolished by an antiserum to CGRP. These observations show that CGRP is involved in the differentiation of dopaminergic olfactory bulb neurons.

In dissociated cell cultures from the embryonic olfactory bulb at 16–17 days of gestation, very few dopaminergic cells were visualized after 2 or 6 days *in vitro* either by immunohistochemical staining with anti-TH antibodies or after [³H]-dopamine uptake and autoradiography (Fig. 1). When olfactory bulb cells and olfactory epithelial neurons were co-cultured, there was a marked (up to fivefold) and rapid (after 2 days *in vitro*) increase in the number of TH-expressing cells. At the same time these cells acquired the capacity to take up exogenous dopamine (Fig. 1). Control experiments showed that cultures of olfactory epithelial neurons did not contain dopaminergic cells.

Most dopaminergic cells had a stellate morphology with short processes (Fig. 2*a, b*) and from their size and shape they were identified as periglomerular interneurons^{9,10}. Other cells, ≤ 10 per culture, had a different morphology: their cell body was larger and at least one of their processes was longer than 10 cell diameters (Fig. 2*c*). These were probably dopaminergic tufted cells that have been described in the bulb previously¹¹. The dopaminergic phenotype, induced in the presence of afferent cells, persisted in co-culture for at least 2 weeks (data not shown), and the induction of dopaminergic properties was observed in cultures from 15-day-old embryos. This latter finding indicates that the presumptive dopaminergic population, although generated by that stage, is silent, at least as far as TH expression and a dopamine uptake are concerned, until a differentiation signal from the afferent cells is provided.

To determine whether the appearance of dopaminergic phenotypes in co-culture results from the selective survival of a neuronal population in the presence of afferent cells, we cultured olfactory bulb cells alone for 4 days *in vitro*, then added olfactory epithelial neurons and assessed the number of

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dopaminergic cells after a total of 6 days *in vitro*. The number of cells exhibiting a dopaminergic phenotype after 6 days *in vitro* was the same whether the olfactory epithelial neurons were added after a 4-day lag or at the time of plating (Fig. 1), indicating that presumptive dopaminergic cells survive in the absence of olfactory epithelial neurons. These results support the hypothesis that afferent cells provide the signals to induce various traits of the dopaminergic phenotype in concert. Moreover, induction by olfactory epithelial cells seems to be specific as it is not evoked in co-culture with hippocampal cells (Fig. 1). Three lines of evidence suggest that this effect is mediated by soluble factors rather than by direct cell interactions: first, TH⁺ cells and olfactory epithelial neurons, identified with a CGRP antiserum as described below, were rarely found in contact in culture; second, a crude homogenate from the embryonic olfactory nerve can replace olfactory epithelial neurons in inducing TH expression and dopamine uptake in olfactory bulb cultures after 2 days *in vitro* (Fig. 1), although the effect is transient, with the number of dopaminergic cells dropping abruptly after 6 days *in vitro* (Fig. 1); and third, the factor(s) responsible for induction in the homogenate are sensitive to trypsin, indicating that they are proteins.

FIG. 1 Effects of olfactory epithelial neurons added at the time of seeding or after 4 days *in vitro* and of an olfactory nerve homogenate, compared with those of hippocampal cells, on the expression of dopaminergic properties (TH-immunostaining (TH) and uptake of dopamine (DA)) in olfactory bulb cultures after 2 and 6 days *in vitro*. All points represent the average of three separate experiments. In each experiment, three coverslips and dishes from sister cultures were counted. Bars indicate $\pm$ s.e.m.; $n=9$.

METHODS. Olfactory bulbs, olfactory epithelium and hippocampus were dissected from 16–17-day-old mouse embryos (Swiss, Charles River). They were dissociated enzymatically²² and cultured in MEM-F10 (1:1) (Gibco) supplemented with glucose, glutamine, sodium bicarbonate and 10% FCS as described previously²³. Cultures of olfactory bulbs were seeded at a density of 10^6 cells per tissue culture dish (35 mm, Falcon) previously coated with polyornithine at $1.5 \mu\text{g ml}^{-1}$, equivalent to six olfactory bulbs per dish. In co-cultures, 10^6 olfactory bulb cells were seeded together with 5×10^5 cells from the olfactory epithelium or from the hippocampus. Control experiments showed that cell density in this range had no influence on the number of dopaminergic cells (data not shown). The distal portions of olfactory nerve were dissected from 15–16-day-old mouse embryos homogenized in a glass homogenizer in CMF PBS at 4 °C and added to the cultures at the time of seeding in a physiological proportion of 1 nerve:2 olfactory bulbs. For immunohistochemistry, the cells were grown on acid-cleaned glass coverslips (40 mm²) previously coated with polyornithine and processed as described in the legend to Fig. 2. The number of TH⁺ cells was assessed by counting whole coverslips. The number of cells taking up dopamine was assessed after autoradiography (see Fig. 2 legend) by counting whole dishes and normalizing the number obtained for 40 mm².

It seemed probable that the expression of dopaminergic properties is regulated by neuropeptides present in the olfactory nerve. Experiments were therefore performed to test for the presence of CGRP and substance P in cultures of olfactory epithelial neurons as both these are present in some afferent fibres of the olfactory nerve^{8,12,13}. Olfactory epithelium cultures plated at a density of 5×10^5 cells per dish contained $6,521 \pm 37$ olfactory epithelial neurons (identified with a MAP1-MAP2-tau antiserum) among which $4,628 \pm 22$ cells were CGRP⁺. Figure 3 is a fluorescence photomicrograph of an olfactory epithelium culture immunostained with a rabbit antiserum to CGRP. CGRP immunoreactivity can be observed in olfactory epithelial cells which appear as fusiform bipolar cells with a club-shaped dendrite and a thinner process originating from the opposite pole of the cell, as already described in culture¹⁴. Substance P immunoreactivity (rabbit anti-substance P, Technogenetics) was weak and present in $\sim 10\%$ of olfactory epithelial neurons (data not shown). To determine whether the peptides are involved in the induction of dopaminergic properties in the bulb, we added CGRP or substance P at various concentrations to olfactory bulb cultures. CGRP at a final concentration of 5×10^{-8} M was found to be optimal for the induction of maximal stimulation.

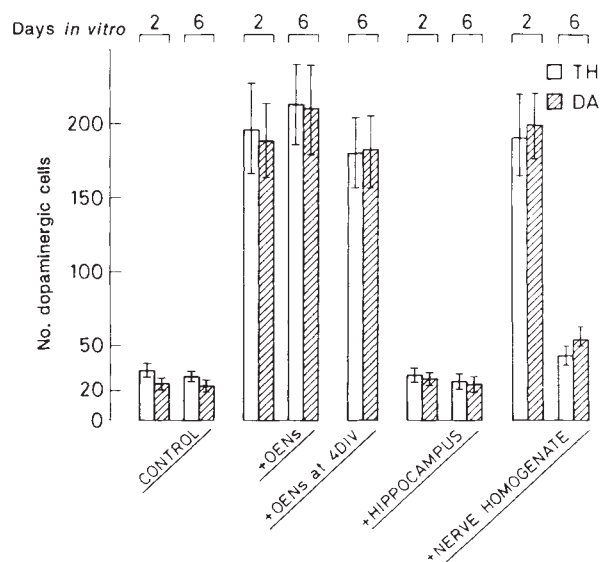
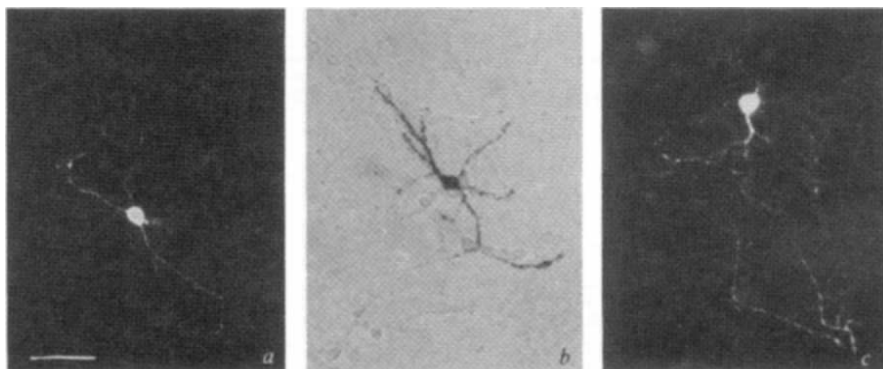


FIG. 2 Fluorescence micrograph of an olfactory bulb culture. TH immunoreactivity is present in periglomerular interneurons (a) and in some tufted cells (c). b, Autoradiography of an interneuron labelled with [³H]-dopamine. Bar, 50 μm .

METHODS. For immunohistochemistry, the cells were fixed in 4% paraformaldehyde in 120 mM phosphate buffer pH 7.4, washed and permeabilized with Triton X-100 (0.3%) and incubated with TH antibodies (Eugene Tech) at a dilution of 1:60. They were then washed and incubated with rhodamine-conjugated secondary antibodies at a dilution of 1:100 (Sigma). Coverslips were mounted in glycerol phosphate and viewed with a Zeiss microscope equipped with epifluorescence. For autoradiography, the cells were incubated for 1 h at 36 °C with [³H]-dopamine (Amersham, specific activity 9–15 Ci mmol⁻¹) at a final concentration of 0.5 μM in PBS supplemented with Ca²⁺ and Mg²⁺ (1 mM each) and 33 mM glucose. They were washed in PBS, fixed in 1.5% glutaraldehyde in PBS, washed extensively,



dried overnight, covered with liquid photographic emulsion (Ilford K5, diluted 1:1 with water) and kept for 4–5 days in the dark before revelation with Kodak D19.

The results, reported in Fig. 4, show that CGRP mimics the effect of olfactory epithelial neurons after 2 days *in vitro*. After 6 days, however, as in the case of the olfactory nerve homogenate, the number of dopaminergic cells dropped to control levels, although a subsequent addition of CGRP caused it to rise again by 8 days *in vitro* (Fig. 4). In contrast to CGRP, substance P had no effect. In many trials with different concentrations of the peptide (10^{-9} – 10^{-7} M), no effect was noticed (Fig. 4) and,



FIG. 3 Chemosensory neurons from the olfactory epithelium immunostained with CGRP antiserum at a dilution of 1:400. Staining was abolished in control cultures incubated with the antiserum together with 10^{-7} M CGRP. Calibration bar, 5 μ m.

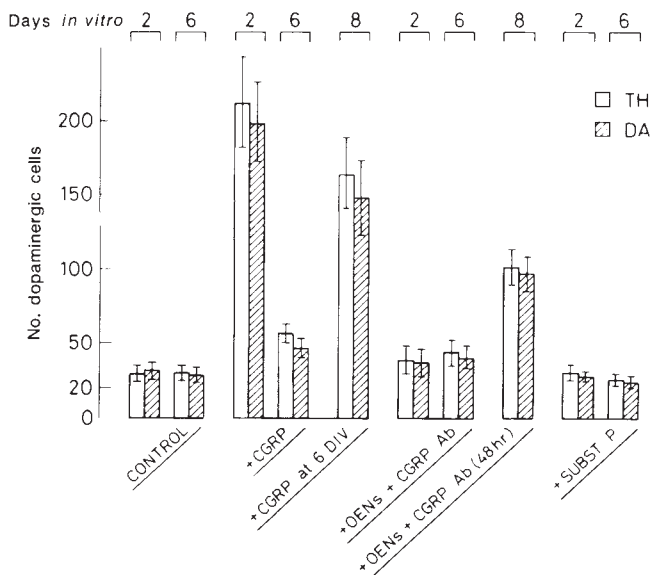


FIG. 4 Effect of CGRP, substance P and CGRP antiserum (CGRP Ab) on the expression of dopaminergic properties in olfactory-bulb cultures and co-cultures, respectively. All points are the average of three separate experiments. In each experiment two coverslips and dishes from sister cultures were counted. Bars indicate $\pm$ s.e.m.; $n=6$.

METHODS. CGRP and substance P (Sigma) were kept as frozen aliquots in sterile water at -80°C . Before use they were diluted in sterile medium in the presence of the peptidase inhibitor bacitracin (0.5 mg ml^{-1}). At this concentration, bacitracin alone had no effect. CGRP or substance P were added at the start of the culture at a final concentration of 5×10^{-8} M and dopaminergic cells were counted after 2 and 6 days *in vitro*. When indicated CGRP was added again after 6 days *in vitro*, dopaminergic cells were counted after 8 days *in vitro*. The CGRP antiserum was added at a dilution of 1:200 at the start of the culture and left for 2 and 6 days *in vitro*. In control experiments, the antiserum was added at the start of the culture for 48 h, washed extensively (three medium changes per hour for 3 h) and dopaminergic cells counted after 8 days *in vitro*.

as expected from these results, there was no sign of additivity or synergism between the two peptides (data not shown). These results show that CGRP induces dopaminergic properties in olfactory bulb cultures and indicate that it might be released by olfactory epithelial neurons. This idea was confirmed by showing that TH expression and dopamine uptake are abolished in co-culture by the addition of antiserum to CGRP at the time of plating (Fig. 4). The cultures remained healthy under such conditions and in control experiments where CGRP antiserum was added for 48 h and then washed extensively, olfactory epithelial neurons still induced the expression of dopaminergic properties at 8 days *in vitro* (Fig. 4). The fact that the number of dopaminergic cells was lower in these experiments could reflect cell loss during the numerous washes after the CGRP antiserum treatment.

In summary, my data show that TH expression and dopamine uptake are induced in culture of embryonic olfactory bulb by the presence of olfactory epithelial neurons and that a strong candidate for mediating this effect is the neuropeptide CGRP. The widespread distribution of CGRP in sensory, integrative and motor systems and in peripheral organs^{8,15} and, in many instances, the lack of coincidence between CGRP immunoreactive fibres and the presence of CGRP receptors¹⁶ indicate several functions for the peptide other than co-mediator or co-modulator at conventional synapses. It may, for instance, act as a muscle trophic factor released from motoneuron terminals as it selectively increases the number of surface acetylcholine receptors^{17,18} and their α -subunit messenger RNA levels¹⁹. Moreover, it controls the rate of desensitization of the receptor²⁰. All these effects are at least in part mediated by the activation of adenylate cyclase^{20,21}. My report, describing a role for CGRP in the differentiation of dopaminergic neurons, raises several questions. At present, it is difficult to assess the instructive versus permissive effect of CGRP on the target neurons and it is important to determine whether induction reflects *de novo* induction or upregulation. It will be interesting to determine whether CGRP acts directly on the presumptive dopaminergic cells or stimulates other cell types to produce other unknown differentiation factors. Finally, it will be a challenge to determine the mechanism underlying the CGRP-mediated induction of dopaminergic properties in the bulb and to understand how the same peptide can be involved in diverse developmental strategies. □

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A histamine-activated chloride channel involved in neurotransmission at a photoreceptor synapse

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COMPARED with the variety of neuromodulatory agents acting through second messenger systems, the number of fast neurotransmitters which directly activate ion channels is limited. Thus, synaptic receptors that act as ligand-gated ion channels have been firmly established only for acetylcholine, glycine, GABA and glutamate, with the first three of these belonging to the same molecular superfamily¹. Recently, however, a possible addition to this list has been suggested as a result of evidence implicating histamine as the neurotransmitter released by a variety of arthropod photoreceptors²⁻⁷. Neurotransmission at this synapse has been studied extensively, particularly in the fly⁸⁻¹². The post-synaptic elements, large monopolar cells, respond to light with a rapid, chloride-mediated hyperpolarization^{8,13,14} that can be mimicked by the application of histamine³. In this report I document some basic properties of the histamine receptors present on large monopolar cells isolated from blowfly optic lobes. The receptor is a ligand-gated chloride channel showing properties consistent with its presumed role of mediating neurotransmission at the photoreceptor synapse.

To analyse the channel, patch-clamp recordings were made from large monopolar cell (LMC) bodies enzymatically dissociated from the first visual neuropile, the lamina. In the whole-cell recording configuration, most (93%, $n = 58$) medium-sized cell bodies (7–12 μm) responded to histamine with large currents (Fig. 1a). Assuming a unitary conductance of 42 pS (see below), the maximum responses corresponded to the simultaneous opening of 100–1,000 channels, consistent with a channel density of $\sim 0.5\text{--}5/\mu\text{m}^2$. It is probable that these channels are mostly extrasynaptic, because in common with other insect neurons, LMC cell bodies receive no synapses. By comparison, in the intact LMC, a saturating light stimulus generates an increase in conductance of up to $\sim 500\text{ nS}$ (ref. 15) corresponding to the simultaneous activation of about 10^4 channels.

With chloride being the only ion equally distributed across the membrane, the reversal potential of these currents was approximately zero ($1.8 \pm 0.9\text{ mV}$, $n = 14$). This shifted systematically with the concentration of chloride ions in the electrode (Fig. 1c), confirming that most of the current was carried by chloride. Even with symmetrical chloride solutions, the histamine-induced current was outwardly rectifying, with slope conductances increasing e -fold per 50–135 mV depolarization (Fig. 1b). As shown below, this rectification is explained by the voltage-dependence of the single channel kinetics.

With an ionophoretic electrode positioned very close to the cell, the response to histamine developed with a latency of less than 0.5 ms, making it unlikely that a second messenger is involved (Fig. 1d). This conclusion is supported by the long-term stability (at least 1 hour) of both whole-cell currents and single channels in isolated outside-out and inside-out patches in the presence of only simple buffered salines. A chloride channel directly gated by histamine has also recently been reported in lobster olfactory receptors¹⁶.

The channels underlying these currents were initially identified in outside-out patches (Fig. 2). Almost invariably (26 of 28 patches) bath histamine ($\geq 10\text{ }\mu\text{M}$) induced rapid channel activity with a chloride-dependent reversal potential. With chloride distributed symmetrically, the single channels had a

conductance of $\sim 60\text{ pS}$ for inward currents (Fig. 3a), and a mean open time of less than 1 ms. Using these criteria for identification, the channels were subsequently investigated using cell-attached or inside-out patches. As would be expected from the estimated channel density, most of these (75%, $n = 87$) contained one or more such channels.

In perfused inside-out patches, the reversal potential showed a similar chloride-dependence to the whole-cell currents (Fig. 1c). In agreement with results obtained for some other chloride channels¹⁷, the channel current-voltage relationship is non-linear, with lower conductances (42 pS) being measured for physiologically relevant outward currents (Fig. 3a). Channel openings were very brief; the mean apparent open time at low histamine concentrations ($\leq 20\text{ }\mu\text{M}$), and a holding potential of

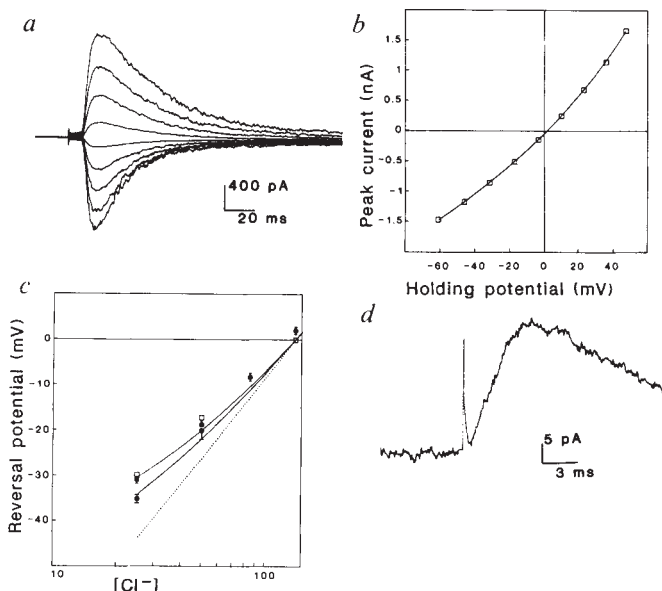


FIG. 1 Whole-cell currents from dissociated blowfly LMCs. *a*, Averaged responses to short pulses of histamine (200 μM , ejected by pressure from a pipette) at various holding potentials between -60 and $+60\text{ mV}$. *b*, Peak currents in *a* plotted against holding potential (corrected for series resistance error and junction potentials²⁴) show an outward rectification. *c*, Reversal potential plotted against $[\text{Cl}^-]$ from a series of experiments similar to *a* but having differing concentrations of acetate (■) or gluconate (●) substituted for chloride in the electrode (bath chloride, 140 mM); each point is the mean of measurements in at least three cells. Open squares, reversal potentials of single-channel currents from inside-out patches, with gluconate being substituted for chloride in the perfusion medium ($n = 4$). Dotted line, Nernst plot for chloride ions. Solid curves, Goldman-Hodgkin-Katz equations assuming permeability ratios of 0.15 and 0.1, respectively, for the substituted anions. *d*, Averaged response to a short pulse of histamine from an ionophoretic electrode positioned 1–2 μm from the cell. The onset of the pulse is indicated by the stimulus artefact, latency is less than 0.5 ms. METHODS. Laminae were dissected out from male blowflies, *Calliphora vicina*, 3–4 days after emergence. To facilitate the dissection, retina and lamina were first pre-digested for 10 min in enzyme solution (1 mg ml^{-1} elastase, nominally Ca-free normal saline). Laminae were then isolated and further digested for 35' at 29 $^{\circ}\text{C}$ in fresh enzyme solution before washing in normal saline supplemented with 10% foetal calf serum. The tissue was then gently triturated, and isolated cells kept at 4 $^{\circ}\text{C}$ for up to 48 h before recording (adapted from ref. 25). Even though the yield consists largely of spherical cell bodies with at most a short neurite, LMCs can be identified by size, as only three other classes of neurons have cell bodies in lamina^{26,27}, and these are either smaller (L4 and L5, $< 7\text{ }\mu\text{m}$) or larger (amacrine, $> 15\text{ }\mu\text{m}$) than the LMCs. Whole-cell recordings were made at 20–22 $^{\circ}\text{C}$ using standard techniques²⁸. Electrodes, pulled from borosilicate glass, had resistances of 5–10 megohm. Electrode solution: 130 mM KCl, 5 mM NaCl, 2 mM MgCl_2 , 5 mM TES, Ca buffered to $5 \times 10^{-8}\text{ M}$ with 1 mM EGTA. Bath contained normal saline (128 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 2 mM MgCl_2 and 15 mM sucrose). In chloride-substitution experiments, appropriate amounts of gluconate/acetate salts were substituted for NaCl (bath) or KCl (electrode). All solutions were buffered to pH 7.1.

-70 mV (near the peak of physiological responses) was 0.56 ± 0.23 ms ($n=21$), coinciding with the time constant of the synapse determined in the intact preparation¹⁰.

A dose-response curve for the channel was constructed by using different concentrations of histamine in the patch electrode (Fig. 3b). The probability of the channel being in the open state increased steeply from ~1% to a maximum value of ~60% over only a fivefold range of histamine concentration. The data were best fitted assuming a high cooperativity (four binding sites) and a dissociation constant (histamine concentration at 50% maximum response) of 6×10^{-5} M. The input-output relationship of the photoreceptor-LMC synapse is one of the steepest reported in the literature (*e*-fold postsynaptic voltage increase for a presynaptic depolarization of 1.5 mV) and also has an exceptionally fast time-constant (0.5 ms) (ref. 10). The combination of low affinity, normally considered a prerequisite for a short open-time¹⁸, and high cooperativity seems well-suited to this role.

As the membrane is depolarized, both the mean open time and the opening rate increase, giving overall, an *e*-fold increase in open probability per 65 mV (± 37 , $n=5$) depolarization (Fig. 4). This can account for the rectification observed in whole-cell currents (Fig. 1), despite the non-linear channel current-voltage relation which rectifies in the opposite direction (Fig. 3a). This voltage dependency, opposite in sign to that of the nicotinic acetylcholine receptor¹⁹, may help shape the transfer characteristic of the photoreceptor-LMC synapse, which is apparently optimized for the coding of natural signals⁹.

Preliminary analysis of open- and closed-time distributions indicates the existence of multiple states, as with other ligand-gated channels. At low histamine concentrations, at least two exponentials were required to fit the open time histograms (Fig. 4a,b). Mean open time increased with increasing histamine concentration, suggesting that the shorter openings may result from single or double occupancies of agonist-binding sites²⁰. Longer openings were frequently interrupted by rapid flickering closures (Figs 3b and 4) which may reflect repeated openings and closures during agonist occupancy²⁰. At low histamine concentrations, closed-time distributions could be fitted by single exponentials (ignoring the rapid flickering closures). At higher concentrations (≥ 30 μ M), however, the channels adopted an obvious bursting behaviour indicative of the appearance of desensitized states²¹.

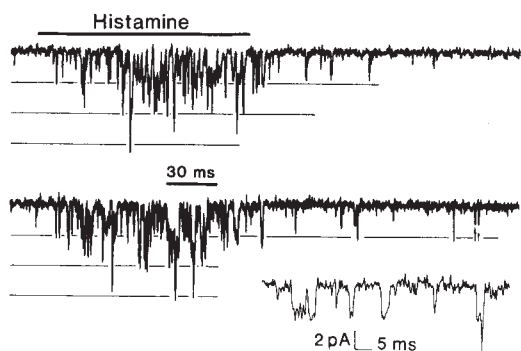


FIG. 2 Channel activity induced by externally applied histamine in outside-out patches. Histamine (50 μ M) was applied during the time indicated, by pressure ejection from a pipette positioned ~20 μ m from the patch. Holding potential was -20 mV, and the bath contained low chloride (25 mM) saline. Horizontal lines, indicating approximate levels of single, double and triple openings, are at 1.9 pA intervals. Many openings are incompletely resolved because of the limited band-width of recording (filtered at 1 kHz, sampled at 10 kHz). Inset, single channels from another outside-out patch on a faster timescale (holding potential -50 mV, normal saline in bath). Electrodes contained the same solution as used for whole-cell recordings (140 mM chloride, see Fig. 1 legend).

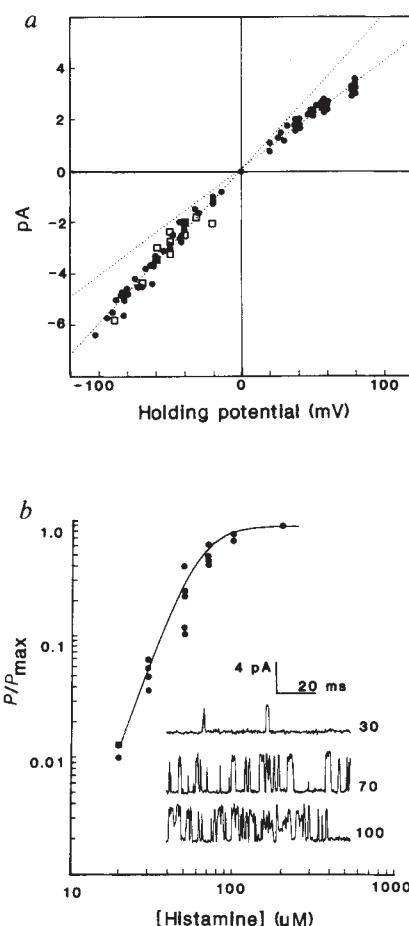


FIG. 3 a, Non-linear current-voltage relationship of the histamine-gated chloride channel. Individual data-points from 13 different inside-out and cell-attached patches (filled circles) and three outside-out patches (open squares) have been pooled (140 mM Cl^- in bath and electrode; histamine concentration, 20–50 μ M). In each case the holding potential is with reference to the reversal potential. Regression lines through the data-points (dotted lines) give a channel conductance of $60.2 (\pm 0.8)$ pS for inward, and $41.8 (\pm 0.9)$ pS for outward currents. b, Dose-response curve of the channel. Normalized open probability (P/P_{max}) at -70 mV plotted against electrode histamine concentration. Data, from 18 patches, are fitted by eye with the equation

$$P/P_{\text{max}} = G^4 / (1 + G^4)$$

where $G = [\text{histamine}] / K_d$ and K_d (the apparent dissociation constant) was estimated as 60 μ M, and P_{max} 60%. Variability in the data also allowed exponents of 3 or 5 (but not 2) to be fitted. Open probability was measured from the idealized records. At histamine concentrations greater than 20–30 μ M, open probability was measured only during bursts of openings; that is, long closures attributable to desensitized states were ignored²⁰. Records were identified as being from single or double channels by the lack of double or triple openings for statistically significant record lengths²⁹. Only at the lowest histamine concentration was there a significant possibility ($p=0.1$, and $p=0.4$ for the square symbol) that the number of channels had been underestimated, leading to an overestimation of open probability and a steeper dose-response curve. Insets, selected channel openings, with 30, 70 and 100 μ M histamine in the electrode.

METHODS. Single-channels were recorded in cell-attached or inside-out configuration with normal saline (see Fig. 1 legend) in bath and either normal saline or normal saline with CsCl substituted for KCl in the electrode to suppress the low-conductance K channels also present in these cells. The use of relatively high resistance electrodes (20–30 megohm after coating with Sylgard and fire-polishing) increased the probability of obtaining single channels, achieved only in inside-out/cell-attached patches. Giga-seals typically in the range 30–100 Gohm were achieved. Records were filtered (4 pole Bessel) at 2–5 kHz and sampled at 17–25 kHz before being idealized using a 50% crossing criterion. Records were analysed using pClamp 4.10 software (Axon Instruments).

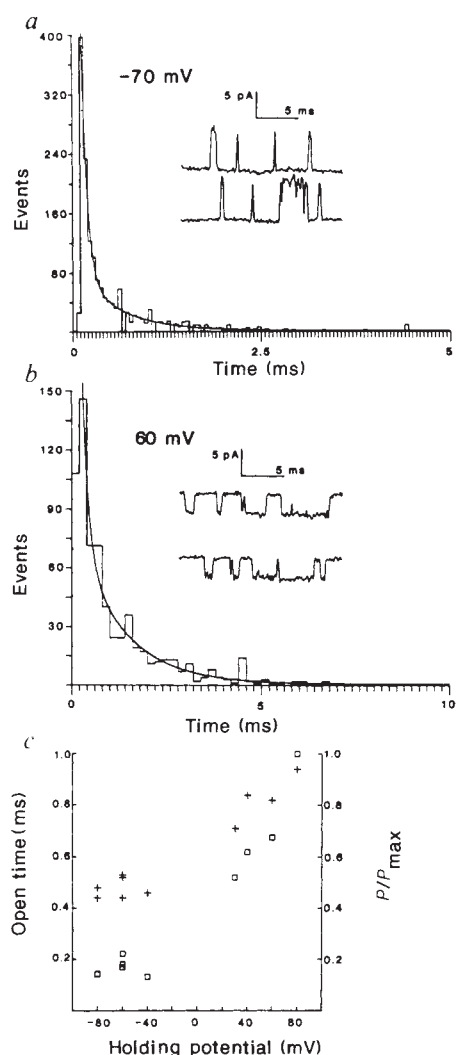


FIG. 4 Open-time distribution histograms from a single inside-out patch at different holding potentials: *a*, at -70 mV (1,645 events), *b*, $+60$ mV (648 events); note the different timescales. The electrode contained $20 \mu\text{M}$ histamine in normal saline; the bath also contained normal saline. Both distributions are fitted by two exponentials: time constants at -70 mV, $69 \mu\text{s}$ (73% of events) and 0.53 ms (27% of events); at $+60$ mV, $160 \mu\text{s}$ (45%) and 1.20 ms (55%). Insets show typical channel openings (-70 mV channel openings, upwards; $+60$ mV openings, downwards). *c*, Voltage dependency of open time (+) and open probability ($\square$) with P normalized to the maximum value, P_{max} , which was 7.1% at $+80$ mV in this inside-out patch. Over the 160 mV range investigated, the mean open time doubled, and the open probability increased sevenfold as the patch was depolarized. Electrode histamine concentration, $20 \mu\text{M}$.

As histamine has now been implicated as the neurotransmitter in several other arthropod eyes including *Drosophila* (P.V. Sarthy, personal communication), moth², cockroach^{2,22}, locust compound eye² and ocellus⁵, barnacle⁶ and *Limulus*²³ and because of its crucial role in visual processing, this receptor type may be expected to be of widespread occurrence. Further studies, in particular of its phylogenetic distribution and its molecular biology, should help to unravel evolutionary relationships and functional specializations within the superfamily of ligand-gated receptors. □

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5-HT₃ receptors are membrane ion channels

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THE neurohormone 5-hydroxytryptamine (5HT or serotonin) exerts its effects by binding to several distinct receptors¹. One of these is the M-receptor of Gaddum and Picarelli², now called the 5-HT₃ receptor, through which 5-HT acts to excite enteric neurons. Ligand-binding and functional studies have shown that the 5-HT₃ receptor is widely distributed in peripheral and central nervous tissue^{3–5} and evidence suggests that the receptor might incorporate an ion channel permeable to cations^{6,7}. We now report the first recordings of currents through single ion channels activated by 5-HT₃ receptors, in excised (outside-out) membrane patches from neurons of the guinea pig submucous plexus. Whereas application of acetylcholine activated predominantly a 40-pS channel, 5-HT caused unitary currents apparently through two channels of conductances of 15 and 9 pS, which were reversibly blocked by antagonists of the 5-HT₃ receptor. Receptors for amine neurotransmitters, including 5-HT₁ and 5-HT₂, have previously been thought to transduce their effects through GTP-binding proteins¹: the direct demonstration that 5-HT₃ receptors are ligand-gated ion channels implies a role for 5-HT, and perhaps other amines, as a 'fast' synaptic transmitter.

5-Hydroxytryptamine has been shown previously to depolarize neurons^{6–11}, including those of the guinea pig submucous plexus^{6–8}, by an action similar to the nicotinic effects of acetylcholine—the responses have brief latency, are due to an increase in cation conductance, desensitize rapidly and are blocked by curare. The effect of 5-HT, however, can be readily distinguished from that of acetylcholine by antagonists such as ICS 205-903 (refs 3 and 6). In submucous neurons, the responses are not mimicked or blocked by compounds active at 5-HT₁ and 5-HT₂ receptors⁶. We made whole-cell recordings from submucous neurons to characterize further this action of 5-HT (Fig. 1). 5-HT (0.5 – $10 \mu\text{M}$) caused a rapidly desensitizing inward current that was carried predominantly by cations because: the reversal potential was the same whether the intracellular solution contained glutamate (3.3 ± 1.3 mV, mean $\pm$ s.e.m., $n = 4$) or chloride (3.6 ± 3.1 mV, $n = 3$); reduction of the extracellular

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sodium concentration from 164 to 80 mM (Hepes substitution) shifted the reversal potential from 1.5 ± 0.9 mV (mean $\pm$ s.e.m., $n = 4$) to -15 ± 1.3 mV ($n = 3$) (theoretical shift is -18.5 mV); and, in three experiments in which the recording pipette contained potassium (replacing caesium), the reversal potential changed from 7 mV to 21 mV when the external potassium concentration was increased from 4.5 to 20 mM. The last result indicates that 5-HT opens channels having a permeability ratio of ~ 2.3 ; this is the same as found in rabbit nodose ganglion⁹. The effect of 5-HT was mimicked by 2-methyl-5-HT ($10 \mu\text{M}$, $n = 3$), which is a somewhat selective 5-HT₃ receptor agonist^{1,3,4}, and blocked by ICS 205-930 (10 – 500 nM) and GR 38032F (10 – $1,000$ nM). The amplitude of the inward current produced by 5-HT (applications for 1 min at 15 min intervals) remained constant ($\pm 5\%$) for up to 3 h of recording, even though the

intracellular solution had no added GTP ($n = 6$). Outward current responses to noradrenaline in these neurons requires a *Pertussis* toxin-sensitive G-protein^{12,13}, and disappear within 15 min after beginning the whole-cell recording unless GTP is included in the intracellular solution (A.S. and R.A.N., unpublished observations). Responses to 5-HT were unaffected ($n = 2$) by pretreatment with sufficient *Pertussis* toxin to prevent G-protein-coupled responses in these cells¹³. These experiments show that acutely dissociated submucous neurons express 5-HT₃ receptors, activation of which increases cation permeability through a mechanism not requiring G-proteins.

We next tested the hypothesis that the 5-HT₃ receptor incorporates an ion channel by applying 5-HT to the extracellular surface of excised membrane patches. Because the nicotinic receptor is known to be a ligand-gated cation channel^{14,15}, we

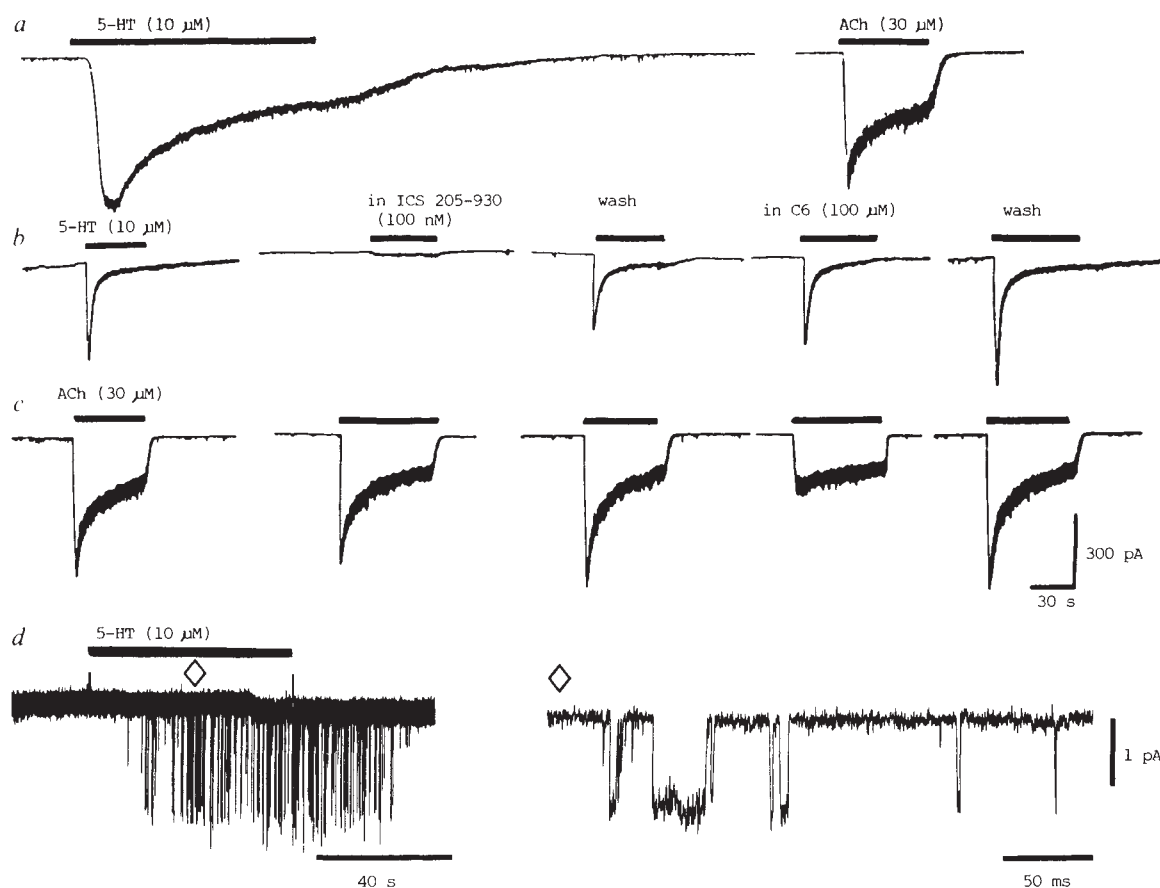


FIG. 1 5-HT causes inward currents. *a*–*c*, Whole-cell recordings showing desensitization of inward currents evoked by both 5-HT and ACh (application period indicated by bars). Note the greater noise associated with the current caused by ACh compared with that activated by 5-HT. *b*, Current induced by 5-HT was blocked by ICS 205-930 (100 nM), recovered substantially after 8 min wash, but was unaffected by hexamethonium (C6, $100 \mu\text{M}$, applied after washing ICS 205-930 for 15 min). *c*, Current caused by ACh was unaffected by ICS 205-930, though much reduced by hexamethonium. *b* and *c* show recordings from the same cell. Holding potential was -72 mV for all recordings; chart recordings shown are filtered at 500 Hz. *d*, Unitary inward currents evoked by 5-HT in an outside-out patch; recordings sampled and played out at 2 kHz, though much reduced by hexamethonium. Right trace shows segment marked by diamond at higher time resolution; sampled at 20 kHz, filtered at 1 kHz. Holding potential, -80 mV.

METHODS. Submucous plexus preparations were dissected from adult guinea pigs^{12,13}; the tissue was incubated at 37°C for 30 – 35 min in a calcium- and magnesium-free Krebs' solution¹³ containing 2 mg ml^{-1} collagenase, triturated, plated onto glass cover slips in Dulbecco's modified Eagle's medium and ganglia allowed to settle for 1 h at 37°C . Experiments were performed 2 – 12 h after dissociation, at 22 – 24°C . Extracellular solutions used for whole-cell and outside-out patch recordings contained (mM): Na^+ ,

167 , Mg^{2+} , 1 ; Ca^{2+} , 1 ; Cl^- , 168 ; glucose, 11 ; HEPES, 10 . Intracellular solutions contained (mM): Cs^+ , 160 ; Na^+ , 24 ; glutamate (or Cl^-), 160 ; Mg^{2+} , 1 ; Ca^{2+} , 1 ; EGTA, 10 ; HEPES, 10 . Patch pipettes used for whole-cell recording had resistances of 2 – $4 \text{ M}\Omega$; potentials are corrected for liquid-junction potentials present before patch formation. ATP (4 mM) was also added to the pipette solution to reduce leakage conductance; internal GTP ($100 \mu\text{M}$) was present in 4 of 10 experiments, but its presence had no obvious effect on the currents evoked by 5-HT or ACh. Single channel currents were filtered at 2 or 3 kHz (4-pole Bessel) and sampled at 10 – 20 kHz for analysis. Electrodes for single-channel recording had resistances of 15 – $40 \text{ M}\Omega$; GTP and/or ATP were present in the pipette solution in some experiments but had no obvious effects on channel activity. The amplitude and open time of unitary events were measured by PCLAMP software (Axon Instruments); only fully resolved events (duration $\geq 0.3 \text{ ms}$) were included for amplitude distributions and open times. The fraction of time for which the channel was open (p_o) was computed from these uncorrected open times. Unless otherwise stated, agonists and antagonists were applied by changing the superfusing solution to one that differed only in the content of the drug(s). 5-HT antagonists used were ICS 205-930 ($[3\alpha\text{-tropylyl}]\text{-H-indole-3-carboxylic acid ester}$) and GR 38032F ($1,2,3,9\text{-tetrahydro-9-methyl-3}[(2\text{-methyl-1H-imidazol-1-yl})\text{-methyl-4H-carbazol-4-one}]$).

compared the action of 5-HT with that of ACh. Fifty-two patches were tested with both 5-HT (1–10 μ M) and ACh (10 μ M): 27 showed unitary inward currents in response to 5-HT (Figs 1*d* and 2), of which 7 also showed currents when ACh was applied, and 10 responded to ACh and not to 5-HT. Thirteen patches responded to neither agonist. Thus, the nicotinic and 5-HT₃ receptors are distinct molecules. 5-HT caused the appearance of unitary currents of two discrete amplitudes, corresponding to conductances of 14.8 ± 0.45 pS ($n = 6$) and 9.2 ± 0.5 pS ($n = 4$) (Figs 3*a* and 4*b*). The reversal potential of the large amplitude currents was 12.0 ± 7.6 mV ($n = 7$). ACh evoked unitary currents of a larger amplitude, the main conductance level being 38.9 ± 0.3 pS, ($n = 3$, Figs 2*c*, *d* and 3*b*). Channels opened by either 5-HT or ACh demonstrated fast openings and bursts of openings, superficially similar to the features of nicotinic channels in other autonomic neurons^{16,17}. The distribution of open times (uncorrected) of the high conductance channels activated by 5-HT was well-fitted by two exponential functions ($\tau_1 = 0.38 \pm 0.1$ ms and $\tau_2 = 5.0 \pm 2.3$ ms, $n = 3$; Fig. 3*c*).

The frequency with which the high- and low-amplitude currents were evoked by 5-HT varied considerably from patch to patch; however, in two of 59 patches where 5-HT evoked unitary currents, only the current of lower amplitude was observed. When 5-HT was applied for up to 10 min, the unitary events of higher amplitude were sustained whereas opening of the channel with lower-amplitude currents became less frequent (Fig. 3*d*). It is not clear whether this contributes to desensitization in whole-cell recording (Fig. 1), because the relative contribution

of currents through large and small amplitude channels is not known.

Channel activity could be reproducibly evoked by repeated applications of 5-HT even up to 5 h after excision of the outside-out patch, suggesting that neither a G-protein nor a diffusible cytoplasmic messenger is necessary. The 5-HT₃ antagonist ICS 205-930 (10–100 nM) markedly reduced the probability of opening of channels in response to 5-HT ($n = 5$) (Fig. 4*a*, *b*); similar results were obtained with GR 38032F (100–1000 nM, $n = 3$) (Fig. 4*a*).

Our results demonstrate that 5-HT₃ receptors are cation channels, placing them in the family of ligand-gated ion channels such as the nicotinic ACh receptor^{14,15}, the excitatory amino-acid receptors¹⁸, and the GABA_A¹⁹ and glycine²⁰ receptors. There are several varieties of, for example, the nicotinic receptor that differ not only in their sensitivity to various antagonists but also in the conductance of the channels^{6,21,22}, and it is possible that the two channel amplitudes observed in the present experiments represent distinct molecular entities; there is some evidence for subtypes of 5-HT₃ receptor on the basis of antagonist affinities^{3,4}. It is also possible, however, that the two conductance levels result from activation of the same receptor molecule.

The neurotransmitters ACh, GABA and glutamate, as well as activating receptors which incorporate an ion channel, also act on distinct receptors that transduce their effects through G-proteins (for example, muscarinic²³, GABA_B²⁴, and one type of quisqualate^{25,26} receptors). Amine transmitters in the vertebrate nervous system have usually been thought to act at receptors

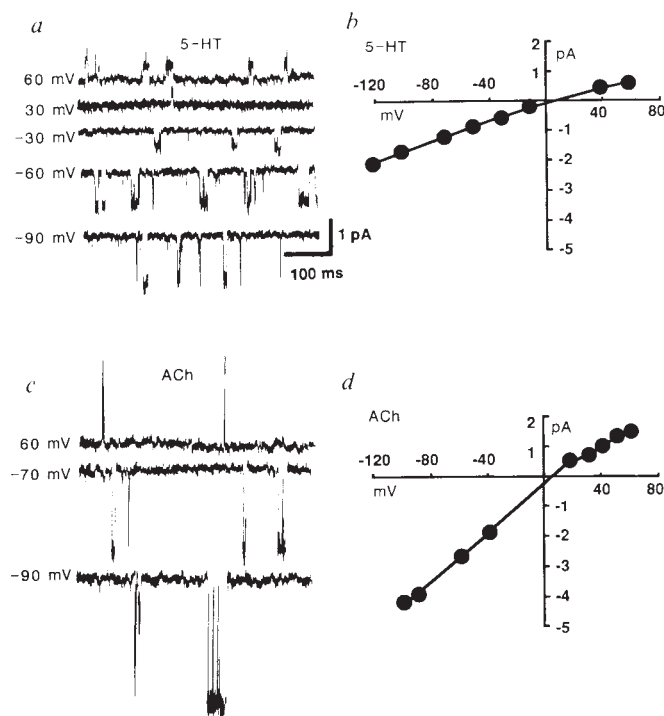


FIG. 2 Single-channel currents evoked by 5-HT (*a*, *b*) compared with those elicited by ACh (*c*, *d*). *a* and *c*, typical channel activities evoked by 5-HT or ACh at the indicated membrane potentials. Calibrations in *a* also apply to *c*. All records were sampled at 10 kHz and filtered at 1 kHz. *b* and *d*, current-voltage relation for the single-channel currents in the presence of 5-HT (*b*, 10 μ M, high-amplitude current illustrated) and ACh (*d*, 10 μ M). Each point is the mean value from 10–20 events; s.e.m. is less than the symbol size. Unitary currents evoked by ACh disappeared quickly during exposure (3–4 min) to agonist, and subsequent applications of ACh evoked no activity for at least 30 min. Therefore, channel activity was evoked by applying (ACh 10 μ M) for periods of 1 s (ACh was ejected from a pipette positioned close to the excised membrane patch), and repeating the applications 2–3 times at each potential. *a* and *c* are from the same patch; lines are drawn by eye.

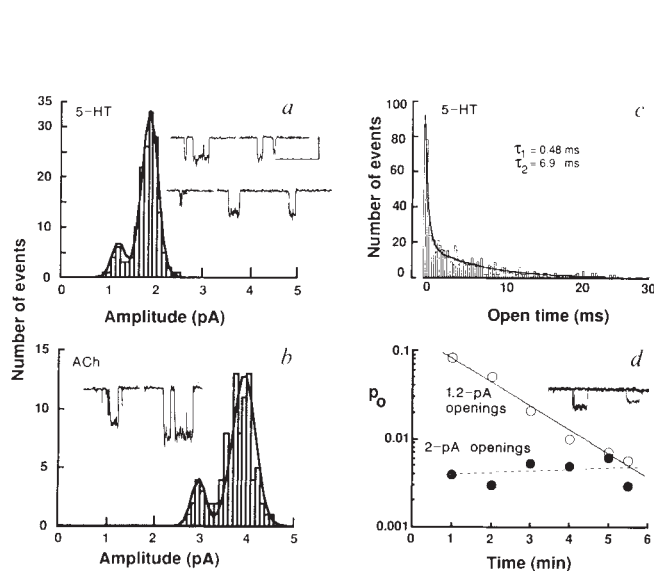


FIG. 3 *a*, Amplitude histogram of channels opened by 5-HT (3 μ M, applied by superfusion) was fitted by the sum of two gaussian distributions with mean $\pm$ standard deviation of 1.2 ± 0.12 pA and 1.9 ± 0.18 pA, respectively; holding potential -112 mV. *b*, Amplitude histogram of channels opened by ACh (3 μ M, applied by superfusion) was also best fit by the sum of two gaussians (3.0 ± 0.13 pA and 3.96 ± 0.28 pA); same patch as *a*, holding potential -112 mV. Calibrations shown in inset of *a* are 2 pA and 50 ms, and also apply to inset of *b*. *c*, Distribution of open times for high conductance channels activated by 5-HT. The continuous line is fitted by a least-squares minimizing method to the sum of two exponentials having time constants τ_1 and τ_2 . *d*, The probability of low-conductance 5-HT channels being open (p_0) decreases with time, whereas openings of high-conductance 5-HT channels remain constant. Data were obtained during continued superfusion with 5-HT (10 μ M), holding potential was -122 mV. Inset shows two superimposed representative traces (100 ms in duration) to illustrate low-amplitude (1.2 pA) and high-amplitude (2 pA) events. p_0 was calculated separately for the high- and low-amplitude events during six successive 1 min periods, the first of which begins about 30 s after commencing superfusion with 5-HT. Amplitude windows were 2.0 ± 0.3 pA and 1.2 ± 0.3 pA.

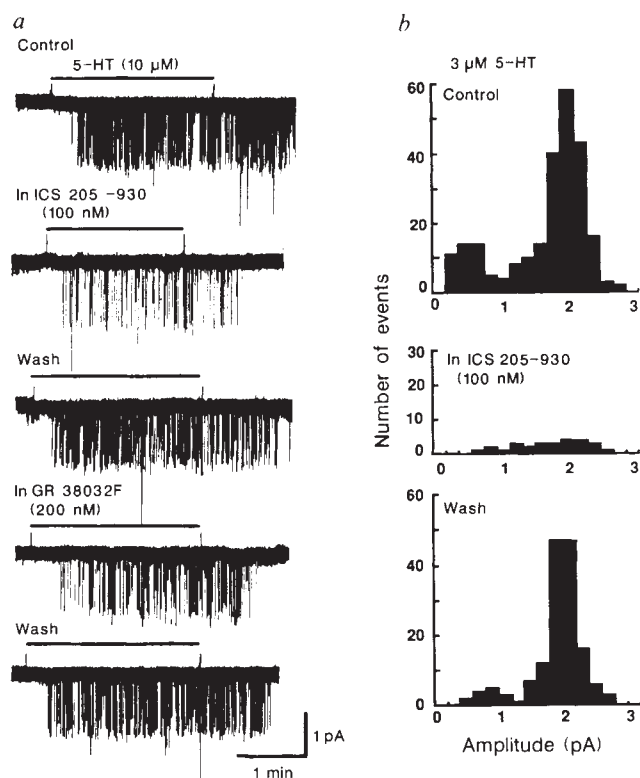


FIG. 4 5-HT₃-receptor antagonists ICS 205-930 and GR 38032F reduce channel opening by 5-HT. *a*, The five traces illustrate unitary activity of one patch caused by 2-min superfusions with 5-HT. 5-HT was less effective to open channels in the presence of the two 5-HT₃ antagonists. Holding potential -80 mV; sampling at 1.6 kHz, filtered at 1 kHz. *b*, Amplitude histograms derived from another patch in the presence of 5-HT (3 μ M, control), in concomitant presence of ICS 205-930 (100 nM), and 8 min after washing out the ICS 205-930. In each case, events were collected during a 6 -min period; holding potential was -112 mV.

which, by activating a G-protein, bring about changes in the excitability of the postsynaptic cell lasting for at least hundreds of milliseconds; 5-HT₁ and 5-HT₂ receptors are examples. The present finding both supports a role for 5-HT as a 'fast' acting neurotransmitter, and raises the question of whether other amines act directly at ligand-gated ion channels. □

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Inhibition of exocytosis by intracellularly applied antibodies against a chromaffin granule-binding protein

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EXOCYTOTIC secretion requires the interaction and fusion of secretory vesicles with the plasma membrane¹⁻³. This process could be mediated by specific recognition molecules acting as intracellular, membrane-bound receptors and ligands⁴. One possible component of such a recognition site on the plasma membrane is a protein of relative molecular mass (M_r) 51,000 (51K) that has been isolated from bovine adrenal chromaffin cells. This protein binds strongly to chromaffin granules, the secretory vesicles of these cells⁵. To determine the function of this membrane-anchored chromaffin granule-binding protein in exocytosis, we tested the effect of intracellularly injected antibodies on secretion. Here we show, by two independent techniques in two different cell types, that antibodies against this protein inhibit exocytosis. In rat pheochromocytoma cell cultures, monospecific antibodies, applied by erythrocyte ghost fusion⁶, impair the release of ³H-noradrenaline. The same antibodies, introduced into individual chromaffin cells through a patch pipette, block exocytosis, as revealed by the measurement of membrane capacitance⁷⁻⁹. These results demonstrate the functional involvement in exocytosis of a plasma membrane protein with high affinity for secretory vesicles.

The 51K chromaffin granule-binding protein (CGBP) was isolated from solubilized plasma membranes of chromaffin cells on the basis of its binding to purified chromaffin granules⁵. In contrast to the soluble cytoplasmic proteins of the chromobindin family¹⁰, this membrane protein binds selectively to chromaffin granules and not to other subcellular organelles; moreover its binding is calcium independent⁵. Antiserum raised against the purified CGBP detects a single protein band of M_r 51K on immunoblots of chromaffin cell membrane but not of chromaffin granule membrane nor of cytosolic protein fractions (Fig. 1, lanes 1a, 2a, 3a). The 51K CGBP is most enriched in subcellular fractions showing the highest activity of the plasma membrane marker enzyme ($\text{Na}^+ + \text{K}^+$) ATPase (data not shown). It is not present in medullary fibroblasts or liver tissue (Fig. 2). The antiserum also recognizes a single protein of M_r 51K in membrane preparations of the pheochromocytoma (PC12) cell line (Fig. 2, lane 5). Low amounts of the 51K CGBP were detected in adrenal cortex preparations (Fig. 2, lane 2), but only a few per cent of the concentration of the 51K CGBP present in adrenal medulla are found in other secretory tissues such as pancreas or whole brain.

For functional analysis, we isolated a subpopulation of antibodies that recognize extramembranous domains of native CGBP (Fig. 1 legend). For control experiments, we raised antiserum against total plasma membrane proteins. These control antibodies recognize various proteins but not CGBP, indicating that CGBP is not a major antigen of the plasma membrane (Fig. 1, lane 1c).

Erythrocyte ghosts were loaded with antibodies and fused with populations of PC 12 cells to introduce the antibodies into the cytoplasm⁶. Despite the high ratio of ghosts to PC 12 cells

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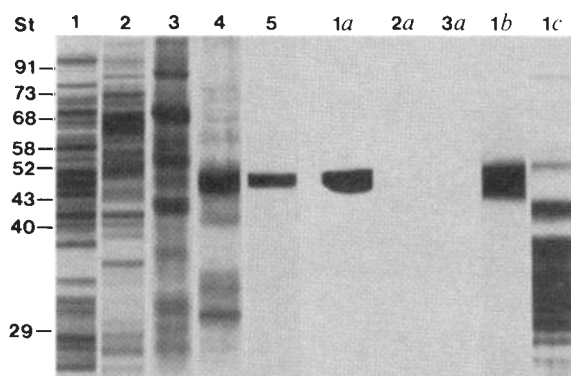


FIG. 1 Preparation and specificity of anti-CGBP antibodies. Lanes 1-4, Coomassie-stained proteins in 10% polyacrylamide gels. Lane 1, crude chromaffin cell plasma membrane fraction (PM); lane 2, chromaffin granule fraction; lane 3, soluble cytoplasmic fraction; lane 4, chromaffin granule-binding proteins; lane 5, silver-stained 51K CGBP used as antigen. Lanes 1a, 2a, 3a, immunoblots of material identical to lanes 1-3 with anti-CGBP antiserum; lane 1b, immunoblot of PM with affinity purified anti-CGBP antibodies; lane 1c, immunoblot of PM with anti-plasma membrane antibodies. Lanes 1b, c are overexposed to demonstrate lack of anti-CGBP titre in anti-plasma membrane antiserum. St, M_r standards, $\times 10^{-3}$.

METHODS. Bovine adrenal medullae were homogenized and adjusted to 10% (w/v) in 0.3 M sucrose, 5 mM EDTA, 10 mM MOPS buffer, pH 7.2. The postnuclear supernatant was centrifuged at 25,000g for 20 min. To obtain the soluble cytoplasmic proteins, the supernatant was centrifuged at 100,000g for 1 h. The 25,000g pellet was layered onto a 1.6 M sucrose cushion. After a 2-h run at 140,000g the pellet was used for the purification of chromaffin granules¹⁹. The interphase of light membranes (crude plasma membranes) was used for the isolation of chromaffin granule binding proteins by passing detergent-solubilized membrane proteins over an affinity column consisting of fixed chromaffin granules⁵. Bound proteins were eluted with a low-pH buffer in the presence of detergent. After separation by SDS-PAGE, the main protein band of 51K was electroeluted and injected subcutaneously into rabbits. Anti-CGBP antibodies were isolated on columns of protein A-Sepharose and of plasma membranes coupled to CNBr-Sepharose. Antibodies directed against total plasma membrane proteins were produced by injecting rabbits with whole plasma membrane fractions and affinity purification on the same columns. For analysis, 100 μ g (lanes 1-3), 10 μ g (lane 4), or 1 μ g (lane 5) of protein per lane was used. On immunoblots, serum was used at a dilution of 1:500, anti-CGBP and anti-plasma membrane antibodies were used at 1 μ g ml⁻¹. Antibodies bound to blots were visualized by the ¹²⁵I-protein A method.

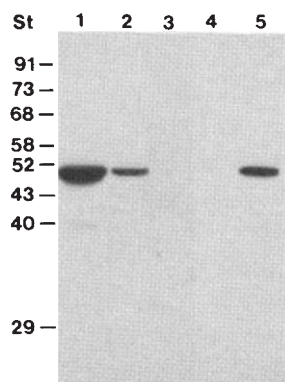


FIG. 2 Distribution of 51K CGBP by immunoblotting. Lanes 1-5, immunoblots with anti-CGBP antiserum; lane 1, adrenal medulla; lane 2, adrenal cortex; lane 3, adrenal medullary fibroblasts; lane 4, liver; lane 5, PC12 cells. The material detected in lane 2 may correspond to medullary impurities in the cortex preparation. St, M_r standards, $\times 10^{-3}$.

METHODS. We separated 100 μ g protein per lane of 25,000g pellets (see Fig. 1) of tissues and cells on 10% polyacrylamide gels. Immunoblotting as Fig. 1. Freshly dissociated medullary cells²⁰ were plated for 4 h on plastic culture dishes. The loosely adherent chromaffin cells were removed and cultured for use in patch-clamp experiments. The strongly adherent cells were kept in culture and used as a source for fibroblasts (lane 3) after cells had formed a monolayer.

(30:1), only a fraction of cells fused successfully. Therefore, we determined the percentage of fused cells ($24 \pm 6\%$; range 13-33%) for each experiment (Fig. 3a). Because the fused cells cannot be separated by various methods such as fluorescence-activated cell sorting, we examined the effect of antibodies on exocytosis in the mixed population of fused and unfused cells.

Secretion of ³H-noradrenaline by cells loaded with control antibodies is the same as that by cells fused with ghosts that do not contain antibodies. Anti-CGBP antibodies, however, have an inhibitory effect on secretion ($P < 0.01$, Student's *t*-test). In four experiments, the extent of inhibition correlated with the number of fused cells. In other words, if the inhibition is attributed only to the population of fused cells, then complete suppression of exocytosis occurs (Fig. 3b,c). This interpretation, based on reproducible but only statistical evidence, was tested by examining the effect of antibodies on individual cells.

Antibodies were introduced into the cytoplasm of single chromaffin cells by the patch-clamp technique in the whole-cell configuration¹¹. To trigger exocytosis, calcium influx was stimulated by depolarizing pulses activating voltage-dependent calcium channels¹². The increase in cell surface area that resulted from the fusion of secretory granules with the plasma membrane during exocytosis was measured electrically as an increase in

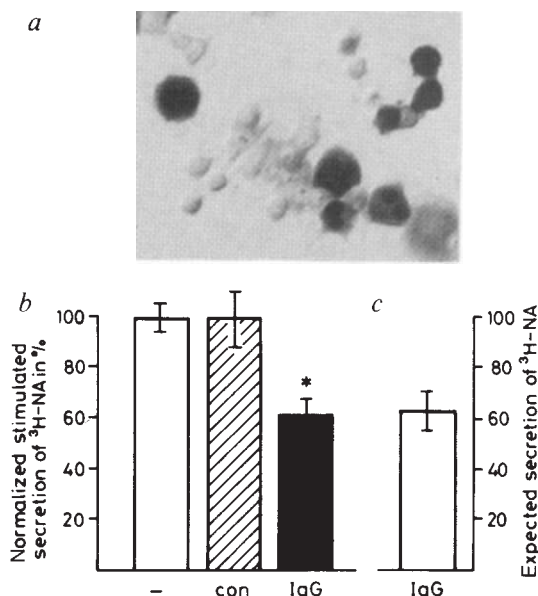


FIG. 3 Inhibition of secretion in PC12 cell populations. a, PC12 cells fused with erythrocyte ghosts containing anti-CGBP antibodies and horseradish peroxidase as marker. The dark peroxidase reaction product allows for the identification of successfully fused cells amidst the clear, unfused cells. b, Secretion of preloaded ³H-noradrenaline from PC12 cells fused with erythrocyte ghosts containing marker only (-), marker and control antibodies (con) and marker and anti-CGBP antibodies (IgG). c, Secretion of ³H-noradrenaline expected under the assumption of a complete inhibition of exocytosis by anti-CGBP antibodies. * $P < 0.01$, Student's *t*-test.

METHODS. Loading of guinea-pig erythrocyte ghosts with antibodies (0.6 mg ml⁻¹) and horseradish peroxidase (1 mg ml⁻¹) was performed as described⁶. The average number of antibody molecules introduced was 2×10^5 per fused cell, as determined by using trace amounts of ¹²⁵I-labelled antibodies. After fusion, aliquots of cells were plated on glass coverslips for determination of the number of fused cells and into plastic tissue culture plates for the measurement of secretion. After an overnight recovery period secretion of preloaded ³H-noradrenaline was stimulated by adjusting the potassium concentration to 55 mM in the external buffer (see Fig. 4) for 15 min at 37 °C. Radioactivity released into the supernatant of unstimulated control cells was measured and subtracted from the respective value of stimulated cells. In each of four independent experiments this difference was normalized to 100% for cells containing control antibodies. This corresponds to an average stimulus-dependent secretion of 16.8% of the preloaded ³H-noradrenaline.

membrane capacitance⁷⁻⁹. In responsive cells, a depolarization for 100 ms to +20 mV reproducibly leads to a 20–80 fF capacitance increase, corresponding to the fusion of about 10–100 granules. Because of the difference in diffusion rates for ions and antibodies¹³ (controlled by including fluorescently labelled antibodies in the pipette) we could measure stimulation-induced capacitance changes before diffusion of antibodies into the cytoplasm. Thus, the exocytotic competence of each individual cell could be established.

Figure 4 shows the typical behaviour of cells perfused with an internal solution containing either control anti-plasma membrane antibodies, anti-CGBP antibodies or anti-CGBP F_{ab} fragments (Fig. 4b–d). In all three cases, shortly after establishment of the whole cell configuration (left traces), depolarization evokes a calcium current (lower traces) and a fast capacitance increase (upper traces). After diffusion of the antibody into the cell (Fig. 4a), responses to stimulation differ drastically (right traces). A 100-ms depolarization still leads to an exocytotic response in cells loaded with control antibodies (three out of three cells). The cell shown in Fig. 4b exhibited considerable calcium current 'run-down' after 20 min, but nevertheless responded with a clear capacitance increase. In seven out of

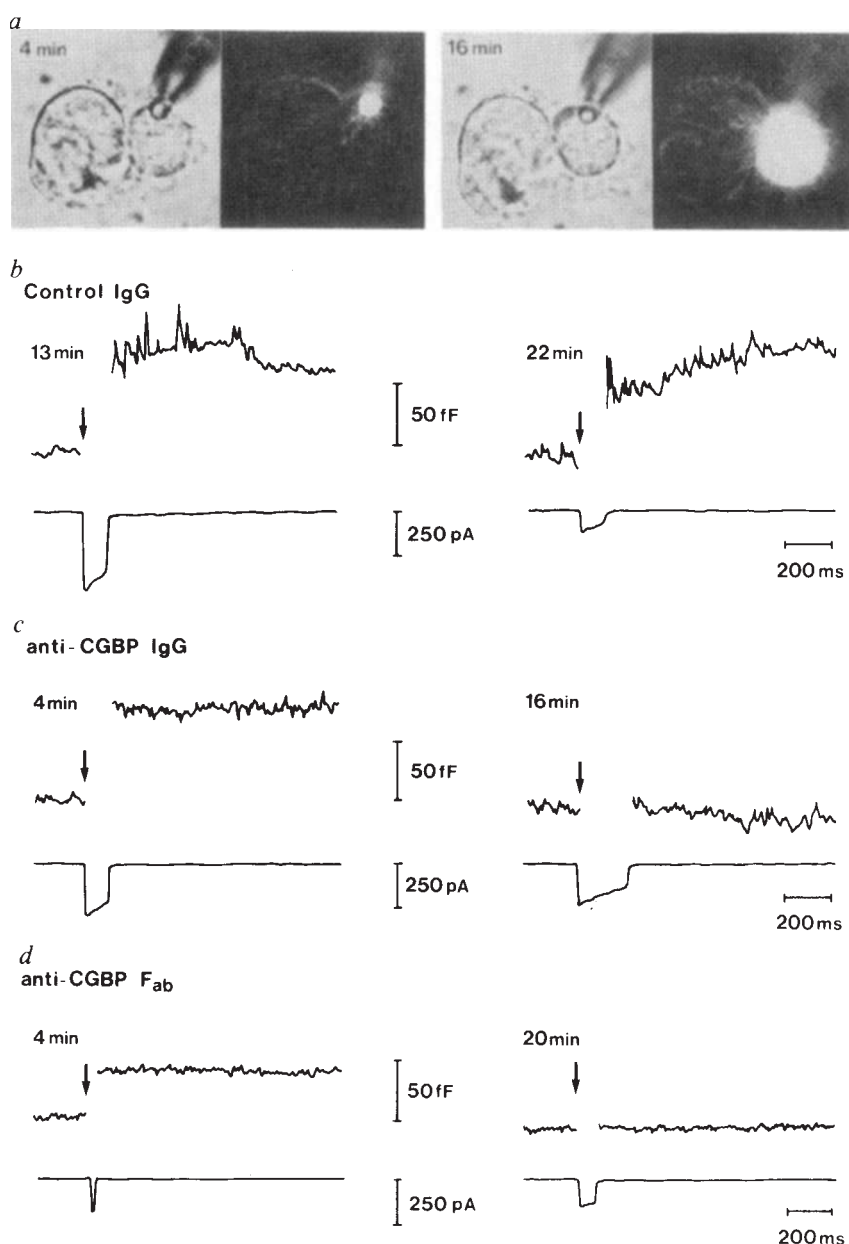
nine cells perfused with anti-CGBP antibodies, however, exocytosis could no longer be triggered after 10–15 min, even by longer depolarizations. The cell shown in Fig. 4c did not respond to a 200 ms depolarization after 16 min, although the calcium current was similar to the current recorded 4 min after breaking into the cell. Essentially the same inhibitory activity was observed using monovalent anti-CGBP F_{ab} fragments. Figure 4d shows a cell responsive to a 10-ms depolarization at 4 min that did not respond to even a 50-ms depolarization after diffusion of the F_{ab} fragments.

These results are in agreement with the conclusion, drawn from the experiments with PC12 cells, that anti-CGBP antibodies can inhibit exocytosis. They further demonstrate that the inhibition cannot be ascribed to blockade of voltage-dependent calcium channels nor to crosslinking of the antigen.

In stimulated secretory systems, vesicles bound or docked to the plasma membrane might represent a pool of immediately releasable vesicles^{14,15}. The close association of vesicles with fusion sites may make them readily available for exocytosis and assure short latencies in stimulus-secretion coupling¹⁶ (less than 10 ms for chromaffin cells; Fig. 4). Other vesicles, directed to and held near the fusion site by cytoskeletal elements, could

FIG. 4 Inhibition of exocytosis in single chromaffin cells. *a*, Bright-field and fluorescence micrographs of a cell loaded with 1:5 diluted FITC-labelled antibody to show diffusion from the pipette into the cell. Left, 4 min; right, 16 min. *b–d*, Membrane current and simultaneous membrane capacitance records taken at indicated times after formation of whole cell configuration from a cell loaded with: *b*, affinity-purified anti-PM antibodies (control); *c*, affinity-purified anti-CGBP antibodies; *d*, monovalent F_{ab} fragments produced from anti-CGBP antibodies. Arrows, beginning of the depolarizing pulses. Scale bars: horizontal, 200 ms; vertical, 50 fF (top bar); 250 pA (bottom bar).

METHODS. Chromaffin cells in 2–8-day-old primary cultures were voltage clamped in the whole cell configuration¹¹ using an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt). To measure cell-membrane capacitance (C_m)⁷⁻⁹, a 15-mV r.m.s. 1 kHz sine wave was superimposed on the holding potential of -75 mV. The resultant membrane current was analysed by a two-phase lock-in amplifier. The imaginary part of the admittance is proportional to C_m after cancellation of whole cell capacitance and series conductance and adjustment of the phase angle between the reference sine wave and the membrane current⁷⁻⁹. Current records were filtered at 80 Hz to eliminate the superimposed sine wave. F_{ab} fragments were prepared according to Porter²¹. Internal solution: 110 mM Cs-glutamate, 20 mM tetraethylammonium-glutamate, 10 mM CsF, 2.5 mM MgATP, 5 mM glucose, 0.5 mM EGTA, 20 mM PIPES-CsOH buffer, pH 7.2, FITC-coupled rabbit-anti-mouse antibody diluted 1:40 and 1 mg ml⁻¹ of the indicated antibodies. External solution: 127 mM NaCl, 5.4 mM KCl, 5 mM CaCl₂, 5.6 mM KH₂PO₄, 4.2 mM NaHCO₃, 0.44 mM KH₂PO₄, 5.6 mM glucose, 1 μ M tetrodotoxin, 10 mM HEPES-NaOH pH 7.2. Micrographs were made using a 100 $\times$ oil-immersion lens.



refill the docking sites. Their interaction with the cytoskeleton supposedly is mediated by fodrin, as anti-fodrin antibodies introduced into detergent-permeabilized chromaffin cells partially inhibit exocytosis¹⁷. The rapid depolymerization of sub-membranous actin filaments observed on stimulation¹⁸ might liberate vesicles tethered in the cytoskeletal meshwork and allow their advance and docking to the plasma membrane.

Our results establish a functional role for the plasma membrane-located 51K CGBP in exocytosis in PC12 and adrenal chromaffin cells. Although the inhibition experiments reported here do not reveal the precise biochemical function of the CGBP, they indicate that the 51K CGBP is part of an intracellular recognition site for chromaffin granules on the plasma membrane. The search for molecules on the chromaffin granules complementary to 51K CGBP could lead to identification of an intracellular receptor-ligand pair suggested by Palade⁴ to be involved in exocytosis. □

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Diversity of $\gamma\delta$ T-cell receptors on murine intestinal intra-epithelial lymphocytes

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THE search for the genes encoding the T-cell receptor (TCR) α - and β -subunits revealed a third gene γ which shares with the α - and β -genes several properties including somatic rearrangement^{1,2}. This gene, together with a fourth rearranging gene δ ^{3,4}, encodes a second type of T-cell receptor, TCR $\gamma\delta$ ⁵⁻⁸. Although TCR $\gamma\delta$ -bearing T cells constitute a relatively minor subpopulation in the thymus and in peripheral lymphoid organs^{8,9}, they are the major lymphocytes of epidermis (dendritic epidermal cells or DEC)¹⁰ and of intestinal epithelium (intestinal intraepithelial lymphocytes or IEL) in mice^{11,12}, suggesting that at least some $\gamma\delta$ T cells are important in the surveillance of a variety of epithelia¹³. It was recently reported, however, that the TCR $\gamma\delta$ on DEC has essentially no structural diversity, implying that the putative ligand is monomorphic¹⁴. As this finding, if generally applicable, poses severe restrictions on the origin of the ligand, we investigated the diversity of the TCR on the second major

epithelium-associated $\gamma\delta$ T cells, namely IEL from mice. We report here that by contrast with the DEC $\gamma\delta$, the IEL $\gamma\delta$ TCR are structurally diverse.

To synthesize DNA encoding the V-J or V-D-J junctional regions of TCR γ - or δ -chains, RNA extracted from IEL was

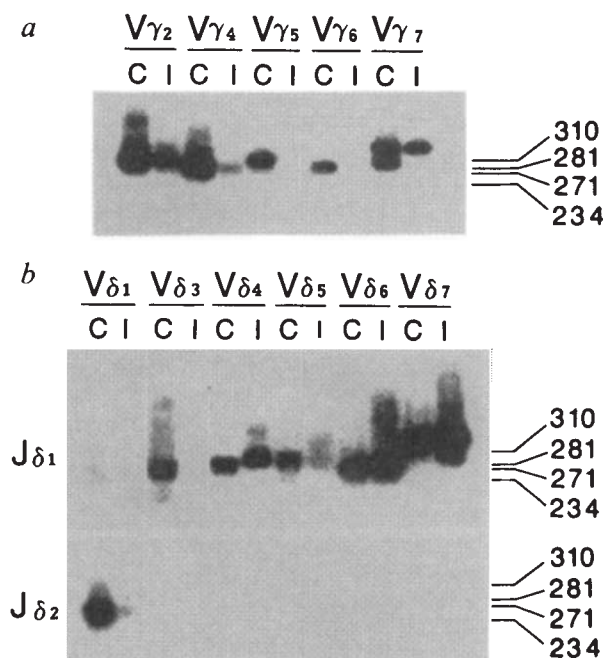


FIG. 1 Detection of TCR γ and TCR δ RNA in IEL by Southern blot analysis of PCR-amplified cDNA. cDNA synthesis was initiated with a C_γ - or C_δ -primer. The sequence of the C_γ primer is shared by all three functional C_γ -gene segments (C_1 , C_2 , and C_4 ; refs 2, 23 & 24). For the subsequent PCR we added a variety of V-specific primers listed in Table I. $V_{\gamma 3}$ was not used because it rearranges only to the non-functional $C_{\gamma 3}$ -gene segment²; $V_{\gamma 1}$ was also not used because the IEL γ protein (relative molecular mass (M_r 34-35K)^{11,12} was clearly different from the $V_{\gamma 1}$ - $C_{\gamma 4}$ protein (M_r 37-42K)^{10,25}. a, γ RNA. The Southern blot of γ -PCR products was hybridized with random-primed $C_{\gamma 1}$ cDNA (*Sfa*NI-*Sfa*NI, 650 base pairs (bp)). The controls (lanes C) were RNA fragments extracted from the following hybridomas: $V_{\gamma 2}$ and $V_{\gamma 4}$, KN6 (ref. 18); $V_{\gamma 5}$, KI129 (ref. 26); $V_{\gamma 6}$, KN25 (ref. 18); and $V_{\gamma 7}$, KN106 (ref. 18). The products from IEL RNA were run in lanes I. b, δ RNA. The Southern blots of δ -PCR products were hybridized with oligonucleotide probes for $J_{\delta 1}$ or $J_{\delta 2}$ as indicated on the left side of the blots. For the controls (lanes C), RNA fragments extracted from the following hybridomas were used: $J_{\delta 1}$, KI129 (ref. 26); $V_{\delta 4}$, KN12 (ref. 18); $V_{\delta 5}$, KN106 (ref. 18); $V_{\delta 6}$, 66-33B (gift of I. Ishida); $V_{\delta 7}$, KN25 (ref. 18). For the $V_{\delta 3}$ control, 0.1 ng of Z68 cDNA¹⁹ cut with *Eco*RI was used. The products from IEL RNA are shown in lanes I.

METHODS. IEL were isolated from adult (15-20-week-old) C57BL/6J mice as described by Petit *et al.*²⁰ and RNA from the lymphocytes in the 50% Percoll fraction was extracted by the guanidinium isothiocyanate/CsCl method²⁷. 10 μ g of RNA was incubated with 0.5 μ M C-primer, 0.5 mM deoxynucleotides, 50 mM Tris-HCl (pH 8.3), 10 mM MgCl₂, 8 mM dithiothreitol, 2 units of human placental ribonuclease inhibitor (Amersham) and 15 units of reverse transcriptase (Seikagaku) in a total volume of 20 μ l. After 45 min at 43 °C, 2.5 μ l of the mixture was brought to 25 μ l in 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 1 μ M V-primer, dNTPs at 250 μ M and 0.5 units of *Taq* polymerase (Perkin Elmer Cetus). PCR cycles were run essentially as described by Saiki *et al.*¹⁵ with 1 min at 92 °C, 2 min at 50 °C and 3 min at 72 °C. A 10- μ l aliquot of the reaction mixture was applied to a 1.5% Agarose gel in 1 $\times$ TBE²⁷, and Southern transfers were performed, as described previously²⁷ on Nitro-plus 2000 cellulose nitrate membranes (MSI). The $J_{\delta 1}$ probe was the oligonucleotide 5'-CTACCGACAACTCGTCTTTGGACAAGGAACCAAGTGAAGTGGAAACCA-3', labelled by Klenow enzyme with the primer 5'-TGGTCCACAGTCAC-3'. The $J_{\delta 2}$ probe was the oligonucleotide 5'-CTCCTGGGACACCCGACAGATGTTTTTGGAACTGGCATAGAGCTCTTTGTGGAGCCCC-3', labelled by Klenow enzyme with the primer 5'-GGGGCTCCACAAAGAGCT-3'. After standard hybridization and washing²⁷, an autoradiogram was generated using the Fujix BA100 Bio-image analyser (Fuji Photo Film Co., Ltd) after exposure for 15 min²⁸.

subjected to the polymerase chain reaction (PCR)¹⁵ (see Table 1 for the synthetic primers used), and subsequently analysed by agarose gel electrophoresis (Fig. 1). Concordant with a previous RNA blotting analysis¹¹, the $V_{\gamma 7}$ -primer gave a strong DNA band of the expected length. DNA bands of appropriate lengths were also observed with the $V_{\gamma 2}$ - and $V_{\gamma 4}$ -primers, but not with the $V_{\gamma 5}$ - or $V_{\gamma 6}$ -primers. As all IEL γ -chains are N-glycosidase-sensitive (data not shown), the $\gamma 2$ chain ($V_{\gamma 2}J_{\gamma 2}C_{\gamma 2}$) which lacks the site for N-linked carbohydrates¹ was not analysed. In the case of TCR δ -chains, a given V_{δ} segment can rearrange to either $J_{\delta 1}$ - or $J_{\delta 2}$ -gene segments⁴: strong PCR bands corresponding to $V_{\delta 4}-J_{\delta 1}$, $V_{\delta 6}-J_{\delta 1}$, and $V_{\delta 7}-J_{\delta 1}$ rearrangements and faint bands corresponding to $V_{\delta 5}-J_{\delta 1}$ and $V_{\delta 1}-J_{\delta 2}$ rearrangements were detected (Fig. 1b).

Both the γ and δ PCR products from the IEL RNA were cloned into a plasmid and their nucleotide sequences determined (Fig. 2). Of the 13 DNA clones obtained from the $V_{\gamma 7}$ PCR

products, 10 had in-frame $V-J$ joints, and three had out-of-frame joints (Fig. 2a), all with N-nucleotide additions¹⁶. Additionally, the joining ends of both the $V_{\gamma 7}$ - and $J_{\gamma 1}$ -gene segments varied among clones. Although the $V_{\gamma 4}$ PCR products also exhibited high junctional variability, all three in-frame clones had a translational termination codon (TAG) indicating that they cannot code for the surface-expressed γ chains. This is consistent with our previous immunoprecipitation analysis¹¹. The nucleotide sequences of the δ PCR products show all but one, pd5-100, to be joined in-frame and therefore have the potential to be expressed on the IEL surface (Fig. 2b). It is evident that all mechanisms responsible for the junctional diversity of TCR δ -genes are utilized in IEL δ chains, namely the use of two known D_{δ} gene segments, the use of D_{δ} gene segments in all three reading frames, the occurrence of N nucleotides in most junctions, and the imprecise joining¹⁷ of the V_{δ} and J_{δ} gene segments.

FIG. 2 $V-J$ junctional sequences of γ and δ transcripts from IEL. a, $V_{\gamma 7}-J_{\gamma 1}$ and $V_{\gamma 4}-J_{\gamma 1}$. The $V-J$ junctional DNA sequences are aligned with V_{γ} germline sequence (Y.T., unpublished observations) or germline $J_{\gamma 1}$ sequence²³. N-regions due to nucleotide additions are shown with normal letters, germline coding sequences are shown in bold letters and heptamer sequences for recombination are indicated in italics. b, $V_{\delta}-J_{\delta 1}$. Junctional DNA sequences are aligned with published germline sequences¹⁹ for $D_{\delta 1}$, $D_{\delta 2}$, and $J_{\delta 1}$ and published V_{δ} sequences with estimated boundaries^{18,19}. N-regions (N_1 , N_2 and N_3) corresponding to the nucleotide additions at VD , DD and DJ junctions are shown in normal letters, germline coding sequences are shown in bold letters and germline heptamer sequences are shown in italics. pd6-92 has the same V_{δ} sequence as M23 (ref. 4). pd7-30 and pd7-33 both have V -region sequences that are slightly different from the other $V_{\delta 7}$ sequences. METHODS. Amplified cDNA from IEL (see Fig. 1) was treated with kinase, purified by 1.5% agarose gel electrophoresis and cloned into the *Sma*I site of the pUC13 vector. After transformation into *E. coli* cells, ampicillin-resistant colonies were screened with labelled $V_{\gamma 7}$ or $V_{\gamma 4}$ probes described previously¹⁸, and plasmid DNA from positive colonies was sequenced by the dideoxy method²⁷ using Sequenase (U.S. Biochemical).

a	V_{γ}				N		$J_{\gamma 1}$			In frame?
	germline $V_{\gamma 7}$	germline $J_{\gamma 1}$								
	-TGT GCC TCC TGG GCT GG	cacaatg.....			cactgtg		AT AGC TCA GGT-			
g7- 19	-TGT GCC TCC TGG GC	CCGAT				AT AGC TCA GGT-			Yes	
g7- 30	-TGT GCC TCC TGG G	GGAGGGGGT				AT AGC TCA GGT-			Yes	
g7- 42	-TGT GCC TCC TGG GC	AT				AT AGC TCA GGT-			Yes	
g7- 51	-TGT GCC TCC TGG GC	GGAAT				AT AGC TCA GGT-			Yes	
g7- 52	-TGT GCC TCC TGG G	TTCTAT				AT AGC TCA GGT-			Yes	
g7- 70	-TGT GCC TCC TGG GCT GG	CCACGG				T AGC TCA GGT-			Yes	
g7- 97	-TGT GCC TCC TGG GCT GG	C				AGC TCA GGT-			Yes	
g7-108	-TGT GCC TCC TGG GCT GG	AT				AT AGC TCA GGT-			Yes	
g7-133	-TGT GCC TCC TGG GCT	ACAT				AT AGC TCA GGT-			Yes	
g7-134	-TGT GCC TCC TGG GCT G	CT				AGC TCA GGT-			Yes	
g7- 56	-TGT GCC TCC TGG GCT GG	TAT				AT AGC TCA GGT-			No	
g7- 57	-TGT GCC TCC TGG GCT	ACGGAT				AT AGC TCA GGT-			No	
g7- 91	-TGT GCC TCC TGG	CCCTAT				AT AGC TCA GGT-			No	
	germline $V_{\gamma 4}$	germline $J_{\gamma 1}$								
	-TGT TCC TAC GGC TAA AG	cacagca.....			cactgtg		AT AGC TCA GGT-			
g4- 7	-TGT TCC TAC GGC TA	G				AGC TCA GGT-			Yes	
g4- 22	-TGT TCC TAC GGC TA	GAAG				AGC TCA GGT-			Yes	
g4- 25	-TGT TCC TAC GGC TA	GCGG				AGC TCA GGT-			Yes	
g4- 16	-TGT TCC TAC GGC TAA A	CCAGATAAGG				CA GGT-			No	
g4- 21	-TGT TCC TAC GG	AG				GGT-			No	
g4- 23	-TGT TCC TAC GGC T	TACGTC				TCA GGT-			No	
g4- 55	-TGT TCC TAC GGC TAA	GAGGA				AGC TCA GGT-			No	
g4- 70	-TGT TCC TAC GGC TAA A	TAGACC				TCA GGT-			No	
b										
	V_{δ}			N_1	$D_{\delta 1}$	N_2	$D_{\delta 2}$	N_3	$J_{\delta 1}$	In frame?
germline $V_{\delta 4}$	-TGT GCT CTC ATG GAG CG									
pd4-203	-TGT GCT CTC ATG GAG CG	CGGGC	GCATAT		TGGGC	CGGAGGGATACGAG	A	ACC GAC AAA-	Yes	
pd4-213	-TGT GCT CTC ATG GAG C	ACACA	TGGC		TCTCAAT	ATCGGAGGGATACGA	CCCTC	CT ACC GAC AAA-	Yes	
pd4-276	-TGT GCT CTC ATG G				GAGG	GGAGGGATACG		CC GAC AAA-	Yes	
pd4-305	-TGT GCT CTC ATG GAG					ATCGGAGGGATA	AAG	ACC GAC AAA-	Yes	
pd4-318	-TGT GCT CTC ATG GAG C	TA	CAT			ATCGGAGGG	CA	T ACC GAC AAA-	Yes	
germline $V_{\delta 5}$	-TGT GCC TCG GGG TAT									
pd5- 17	-TGT GCC TCG GGG TAT	TGG	ATATC			GAGGGATACGAG	CTTGG	CC GAC AAA-	Yes	
pd5- 86	-TGT GCC TCG GGG TAT	CCC	GCAT			CGGAGGGA	G	CT ACC GAC AAA-	Yes	
pd5-100	-TGT GCC TCG GGG	CTC	CATAT	GGCA	ATCGGAGGGATAC	AGG		ACC GAC AAA-	No	
pd5-187	-TGT GCC TCG GGG	GAA	ATAT			GG	CCCT	CT ACC GAC AAA-	Yes	
pd5-238	-TGT GCC TCG GG	CTTC				GAGGG	CCCCA	CT ACC GAC AAA-	Yes	
germline $V_{\delta 6}$	-TGC GCT CTC TCG GAA CT									
pd6- 32	-TGC GCT CTC TCG GAA CT	TGGA	ATAT			ATCGGAGGGATACG	T	CT ACC GAC AAA-	Yes	
pd6-138	-TGC GCT CTC TCG GAA CT	G			ACC	AGGG		CT ACC GAC AAA-	Yes	
pd6-201	-TGC GCT CTC TCG GAA C	GTGGGAGGCCACCA	CATATC		GG	AGGGATACGAG	CTGGG	C GAC AAA-	Yes	
pd6-203	-TGC GCT CTC TCG G	TC	GT		T	ATCGGAGGGATACGAG		CT ACC GAC AAA-	Yes	
pd6-204	-TGC GCT CTC TCG GAA CT				G	TCGGAGGGATACG	GGG	CT ACC GAC AAA-	Yes	
pd6- 92*	-TGT GCT CTC TGG GAG C	CT	TAT			ATCGGAGGGATACG	GA	CT ACC GAC AAA-	Yes	
germline $V_{\delta 7}$	-TGT GCT ATG G									
pd7- 37	-TGT GCT ATG G		ATC		C	ATCGGAGGGATACGAG	CTGACG	GAC AAA-	Yes	
pd7- 43	-TGT GCT ATG G	AAC	GTGG		G	CGGAGGG	AC	ACC GAC AAA-	Yes	
pd7- 51	-TGT GCT A	GGAGGG	ATATC			GGAGGGATACGAG		CT ACC GAC AAA-	Yes	
pd7-152	-TGT GCT ATG		GTGG		G	CGGAGGGATACGAG	AGG	CT ACC GAC AAA-	Yes	
pd7- 30#	-TGT GCT AT	A				GGAGGGATACGA	CT	C GAC AAA-	Yes	
pd7- 33#	-TGT GCT A	GA				ATCGGAGGGATAC	ACC	CT ACC GAC AAA-	Yes	
germline $D_{\delta 1}$	cactgtg GTGGCATATCA cacaggt.....									
germline $D_{\delta 2}$	caccgtg ATCGGAGGGATACGAG cacagtg.....									
germline $J_{\delta 1}$	agctgtg CT ACC GAC AAA-									

TABLE 1 PCR primers

Region	Name	Sequence 5'-3'	Position	Ref.
C_γ	STP. 120	CTTATGGAGATTGTTCAGC	139-145	1
$V_{\gamma 2}$	STP. 121	CGGCAAAAAACAAATCAACAG	37-43	1
$V_{\gamma 4}$	STP. 073	TGTCCTTGCAACCCCTACCC	49-56	29
$V_{\gamma 5}$	STP. 094	TGTGCACTGGTACCACTGA	35-42	23
$V_{\gamma 6}$	STP. 107	(GGAA)TTCAAAGAAAACATTGTCT	55-62	23
$V_{\gamma 7}$	STP. 102	AAGCTAGAGGGGTCTCTGC	18-24	30
C_δ	STP. 110	CGAATCCACAATCTTCTTG	158-165	3
$V_{\delta 1}$	STP. 111	(GGA)ATTGAGAAGGCAACAATGAAAG	79-86	4
$V_{\delta 3}$	STP. 119	TTCTGCTATTGCCTCTGAC	65-72	19
$V_{\delta 4}$	STP. 075	CCGCTTCTCTGTGAACCTCC	61-68	18
$V_{\delta 5}$	STP. 082	CAGATCCTCCAGTTCATCC	42-49	18
$V_{\delta 6}$	STP. 113	TCAAGTCCATCAGCCTTGTC	72-78	3
$V_{\delta 7}$	STP. 076	CGCAGAGCTGCAGTGTAACT	18-25	18

The position of the nucleotide sequence of the primer is indicated by the corresponding amino-acid number counted from the putative N-terminal cleavage site in each reference. For the $V_{\delta 6}$ primer, a sequence common to p12, Z53 and Z49 was chosen. The 3' 15 bases of this primer are also common to M23 (ref. 4).

The amino-acid sequences deduced from the junctional nucleotide sequences indicate that IEL $\gamma\delta$ TCR would have a high degree of structural diversity in the V - J junctional regions (data not shown); but diversity is not limited to these regions because the $V_{\gamma 7}$ -coded γ -chain can pair with either the V_4 , V_5 , V_6 or V_7 δ -chain. This diversity of the IEL $\gamma\delta$ TCR is reminiscent of that observed for the $\gamma\delta$ TCR expressed on the thymocytes of adult mice^{18,19}. The IEL $\gamma\delta$ TCR, however, clearly comprise a unique subset distinct from those on adult thymocytes which use $V_{\gamma 4}$ and $V_{\delta 5}$ gene segments predominantly.

The $\gamma\delta$ TCR expressed on DEC, the other known epithelium-associated $\gamma\delta$ T-cell subset, utilize a single V_γ ($V_{\gamma 5}$) and a single V_δ ($V_{\delta 1}$) gene segment and have no junctional diversity¹⁴. This suggests that the ligand for DEC $\gamma\delta$ TCR is monomorphic unlike those of $\alpha\beta$ TCR¹⁴. By contrast, IEL certainly have the capacity to recognize structurally diverse ligands with their highly diverse $\gamma\delta$ TCR. This, plus the fact that IEL are CD8-positive^{20,21} strongly suggests that their ligand is composed of a structurally variable peptide presented by a class I or class I-like protein of the major histocompatibility complex (MHC). The high level of diversity concentrated in the V -(D)- J junctions is consistent with the recognition of variable peptides, if the folding of polypeptide chains is similar for TCR $\gamma\delta$ and immunoglobulin molecules²². The origin of the postulated peptides is a matter of speculation. One possibility is that they originate from a relatively large set of self proteins whose syntheses are induced when the epithelial cells are under stress. Another possibility is that the peptides arise from viruses, bacteria and other microorganisms that are prone to infect the intestinal epithelium cells. The preferential usage of the $V_{\gamma 7}$ segment may reflect its affinity for a limited number of class I or class I-like protein(s) that may be expressed on intestinal epithelial cells. □

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Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin

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IN addition to having profound effects on embryonic pattern formation¹⁻⁵, retinoic acid (RA) has striking effects on differentiation and maintenance of epithelial cells *in vivo* and *in vitro* (reviewed in refs 6 and 7). Skin is a major target organ for retinoids both in its normal⁶⁻⁹ and pathological states¹⁰. The discovery of two human nuclear receptors for RA (hRAR α and hRAR β) acting as transcriptional RA-inducible enhancer factors¹¹⁻¹⁴ has provided a basis for understanding how RA controls gene expression^{15,16}. To investigate the specific role that RARs might play during development and in adult tissues, we have cloned the mouse RAR α and RAR β (mRAR α and mRAR β). Their amino-acid sequences are much more homologous to those of hRAR α and hRAR β , respectively, than to each other, which suggests strongly that RAR α - and β -subtypes have different functions. Most interestingly we have discovered a novel RAR subtype (mRAR γ) whose expression in adult mouse seems to be highly restricted to skin, whereas RAR α and RAR β are expressed in a variety of adult tissues. Furthermore, both mRAR α and mRAR γ RNAs are readily detected in undifferentiated F9 embryocarcinoma (EC) cells, whereas mRAR β messenger RNA is induced at least 30-fold in RA-differentiated F9 cells.

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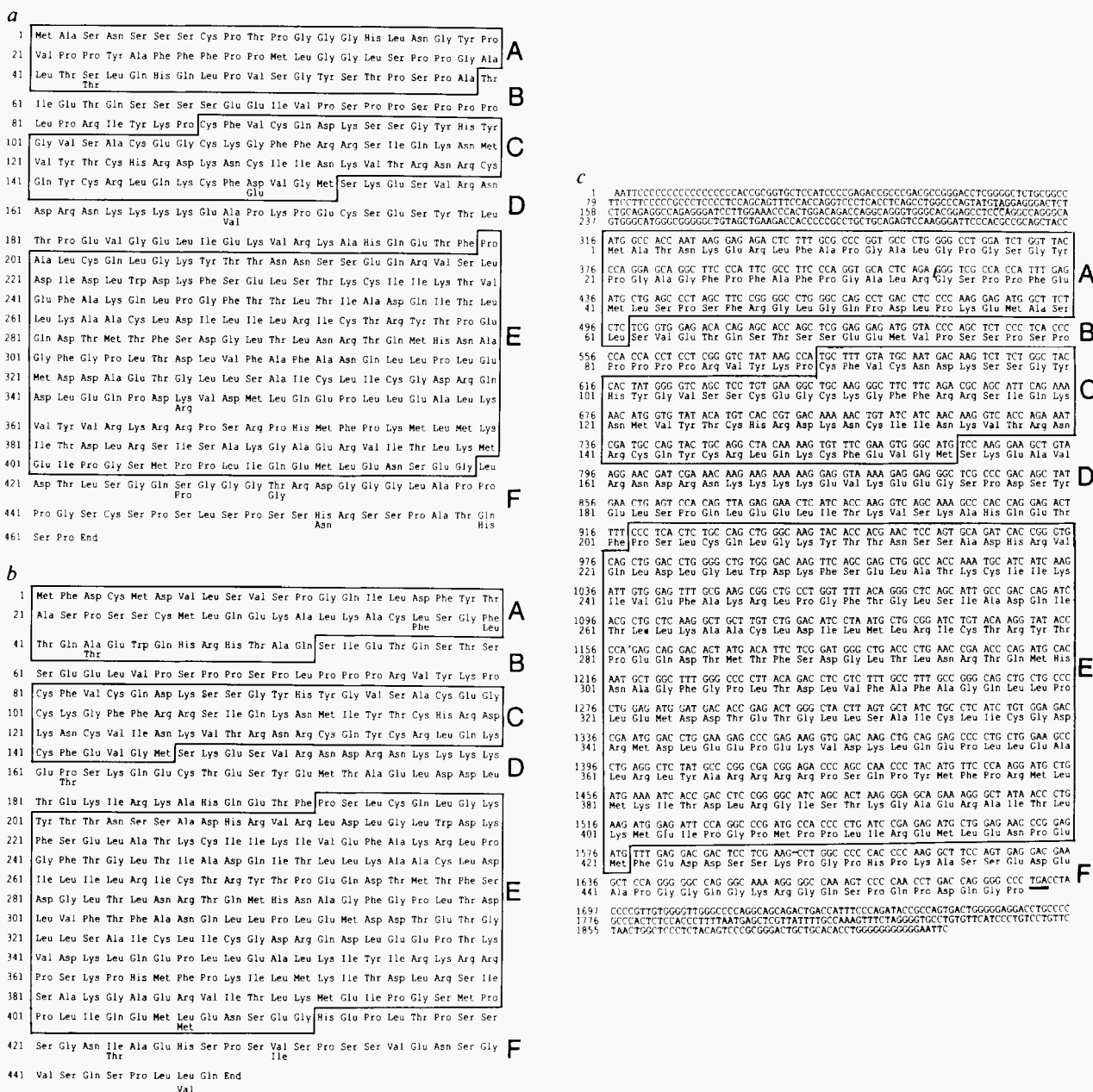


FIG. 1. Amino-acid sequences of mRAR α (a) and β (b), and nucleic-acid and deduced amino-acid sequences of mRAR γ (c). Nucleotides and amino acids are numbered on the left side of each sequence. An in-frame termination codon (positions 145–147) in the 5'-untranslated region of mRAR γ cDNA is underlined in c. Regions A, C and E are boxed, and the location of regions A–F is indicated on the right side of each sequence (see text). Amino acids differing between mRAR α and mRAR β and their human cognates are indicated in a and b under the murine sequences. The termination codon at the end of the mRAR γ sequence is underlined in c and the ends of mRAR α and mRAR β amino-acid sequences are indicated in a and b. The sequences of mRAR α and mRAR β cDNAs are available upon request.

METHODS. Approximately 2×10^6 recombinant phage from an 11.5-day, randomly-primed, mouse-embryo λ gt10 cDNA library (donated by B. Galliot and D. Duboule) were screened with purified hRAR α and hRAR β cDNA probes derived from clones hRAR α O and hRAR β O^{11,13} and ³²P-labelled by random priming²⁴ to a specific activity of 10^9 c.p.m. μ g⁻¹ of DNA. Plaque lifts onto nitrocellulose filters and treatment of the filters before hybridization were carried out as described previously²⁴. Hybridization was carried out at 37 °C for ~24 h in 5 $\times$ SSPE (0.75 M NaCl, 50 mM NaH₂PO₄ and 5 mM EDTA, pH 7.4), 40% formamide, 0.2 mg ml⁻¹ sheared and denatured salmon-sperm DNA.

and $1 \times$ Denhardt's reagent²⁴. The most stringent wash was done in $2 \times$ SSPE plus 0.1% SDS/0.06% sodium pyrophosphate (NaPPi) at 50 °C for 20 min. Filters were exposed for 36 h at -80°C (one intensifying screen, Kodak XRO-5 film). Positive clones were rescreened under the same conditions and phage DNA was prepared from the clones which remained positive. These clones were divided into three groups based on Southern blot hybridization data with hRAR α and hRAR β cDNA probes and restriction enzyme digest patterns. Selected cDNAs from each group were subcloned into either pTZ19R, or pEMBL18 and 19 vectors; single stranded DNA was prepared²⁴ and sequenced using the dideoxy chain-termination procedure²⁵. Two clones which contained the entire open reading frame of mRAR γ and mRAR β were sequenced either on both strands (mRAR γ , sequence shown in panel c), or on one strand (mRAR β , panel b; any ambiguity was resolved by sequencing on the other strand). The mRAR α sequence was derived from two clones which were sequenced on both strands and overlapped between amino acids 64 and 283 of the sequence displayed in panel a. A mRAR α cDNA clone containing the entire open reading frame was subsequently constructed in pSG5 (ref. 21) by using the unique *Bsu*361 site located in the DNA sequence encoding amino acids 182–184.

An 11.5-day-old total mouse embryo λ gt10 complementary DNA library was screened with hRAR α and hRAR β cDNA probes: 81 clones were isolated, of which two sets were identified as mRAR α and mRAR β on the basis of a 98% homology of their cDNA-deduced amino-acid sequence with that of hRAR α and hRAR β , respectively (Fig. 1a, b). Less homology with RAR α or RAR β was found for a third set of clones, although the deduced amino-acid sequence (Fig. 1c) was obviously related to both of them (Fig. 2; the A-F regions in Figs 1 and 2 were as previously defined in refs 11, 13 and 16). This new member of the mouse RAR subfamily was designated mRAR γ . The greatest amino-acid sequence similarities among the three mRARs were found in the regions corresponding to the DNA-binding domain (region C, 95%) and the ligand-binding domain (region E, 85% identity between mRAR α and mRAR γ , and 90% identity between mRAR β and either mRAR α or γ), suggesting that mRAR γ recognizes the same responsive element and binds the same ligand as mRAR α and mRAR β (see below). Region B is also conserved (75%, 86% and 79% identity between mRAR γ and mRAR α , mRAR γ and mRAR β , and mRAR α and mRAR β , respectively), whereas no conservation was seen in this region when comparing nuclear receptors that bind different ligands (ref. 16 and refs therein). The D region, which is not conserved across the nuclear-receptor family of a given species and may act as a hinge region¹⁶, is less conserved among mRARs. Both the N- and C-terminal segments of region D, however, are highly conserved, although the central segment is not (hatched box in Fig. 2). No significant similarity was found between mRARs in region A (encoded in an exon different from that encoding region B, see ref. 13), nor in region F, both of which also vary within a given species between the different nuclear receptors¹⁶.

By contrast, there is an almost complete conservation of amino-acid sequence between the A regions of hRAR α ^{11,12} and mRAR α (98%), and hRAR β ^{13,14} and mRAR β (94%), and between the corresponding F regions (90% for hRAR α and mRAR α , and 92% for hRAR β and mRAR β). Similarly, the entire D region of a given RAR subtype is conserved across

species (98% identity between both hRAR α and mRAR α , and hRAR β and mRAR β). This high degree of conservation of the A, D and F regions for a given RAR subtype contrasts with the lack of or lower conservation of the same regions among the various RARs in a given species (see above) and also with the lower conservation of regions A/B, D and F for a given steroid-hormone receptor across species^{16,17}. Thus the A, B, D and F regions may have specific functions not performed by the C and E regions, but necessary for the three RARs to exert their specific physiological roles. Note that the A/B regions of the oestrogen and progesterone receptors have been implicated in specific transcriptional transactivation of some target genes^{16,18-20}.

When mRAR α , mRAR β and mRAR γ cDNAs were expressed²¹ in HeLa cells together with a reporter plasmid (TRE3)₃-tk-CAT containing a RA-responsive element (Fig. 3), all three receptors responded similarly to all-trans RA. As was the case for hRAR α and hRAR β ^{11,13}, retinol was much less efficient at the same concentrations (data not shown). No obvious difference was observed between the dose responsiveness of mRAR α and mRAR β , in contrast with results obtained previously with human chimaeric RAR α and RAR β ¹³. This may be due to the use of a less sensitive responsive element in the present study.

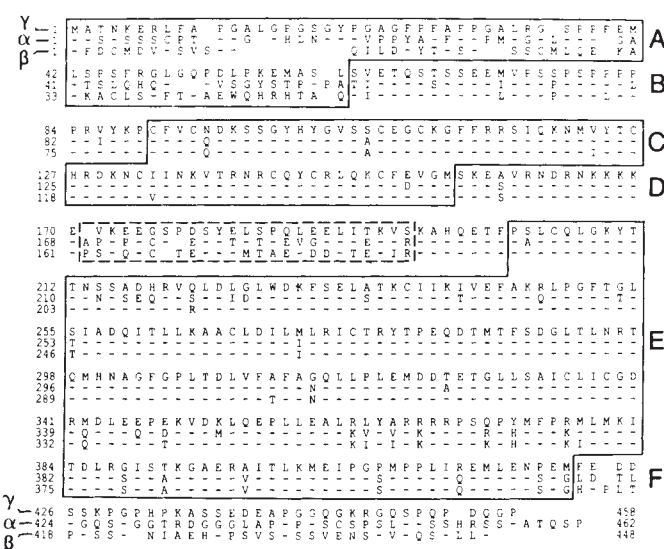


FIG. 2 Amino-acid alignment of the mRAR α , mRAR β and mRAR γ (as indicated). The single letter amino-acid code is used, and the number of the last amino acid in each sequence is given at the end of the alignment. Numbers on the left side correspond to the first amino acid in a given line. Regions A, C and E are boxed and regions A-F are designated with capital letters on the right side of the figure. The non-conserved part within region D is boxed with a hatched line. Gaps have been introduced to obtain the optimal alignment of the mRAR γ sequence with that of mRAR α and mRAR β . Dashes represent mRAR α and mRAR β amino acids which are identical with those of mRAR γ .

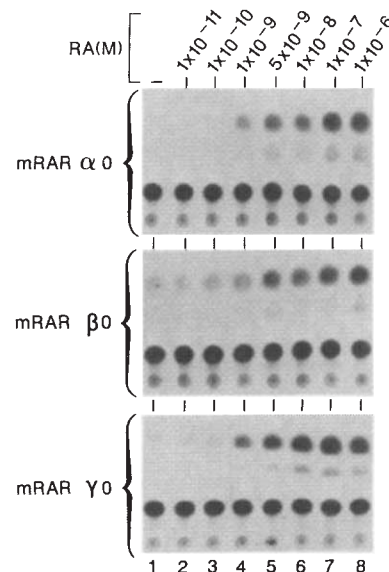


FIG. 3 RA-dependent transcriptional trans-activation by mRARs transiently expressed in HeLa cells. HeLa cells were co-transfected as described^{11,13} with pSG5-based expression vectors²¹ containing the entire cDNA of either mRAR α (mRAR α 0), mRAR β (mRAR β 0) or mRAR γ (mRAR γ 0), and a reporter plasmid, (TRE3)₃-tk-CAT, carrying a synthetic RA-responsive element (RARE). After transfection (24 h), cells were fed with media containing increasing concentrations of RA as indicated ($0-1 \times 10^{-6}$ M, lanes 1-8, respectively) and collected 48 h after transfection for determination of CAT (chloramphenicol acetyltransferase) activity.

METHODS. HeLa cells ($\sim 10^6$ per dish) were co-transfected with 0.5 μ g of a given mRAR expression vector, 2 μ g of reporter plasmid and 2 μ g of pCH110 (Pharmacia, a β -galactosidase expression vector used as an internal control to normalize for variations in transfection efficiency). The total amount of transfected DNA was adjusted to 20 μ g by addition of carrier DNA (BSM13+). Cell culture media, treatment of cells, preparation of cytosolic extracts, and CAT assays were carried out as previously described^{11,13}. The reporter plasmid (TRE3)₃-tk-CAT (ref. 26) contains a trimer of a synthetic RARE (5'-AGCTTAGGTCAGGACGTGACCTT-3') inserted in pBLCAT8⁺ (ref. 27) upstream of the tk promoter. mRAR α 0 was constructed by inserting the reconstructed mRAR α cDNA (see Fig. 1) into the *Eco*RI site of pSG5 (ref. 21), mRAR β 0 was obtained by inserting the *Eag*1/*Bam*HI fragment of mRAR β cDNA into the *Bam*HI site of pSG5 with the help of a *Bam*HI/*Eag*1 adaptor, and mRAR γ 0 was constructed by ligating the *Eco*RI-flanked mRAR γ cDNA (Fig. 1c) into the *Eco*RI site of pSG5.

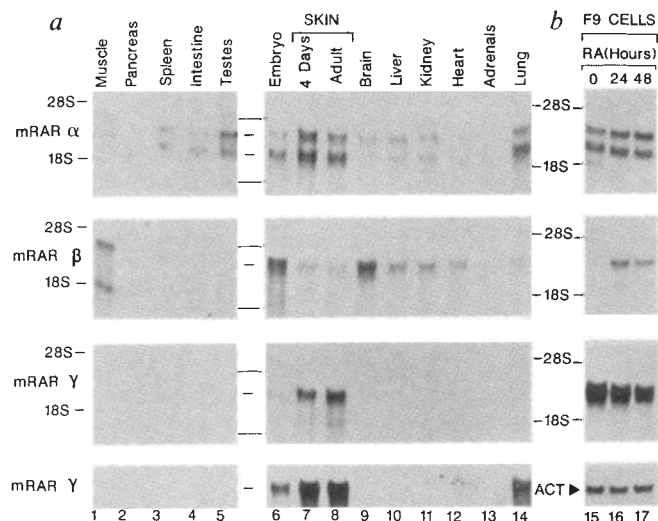


FIG. 4 Northern blot analysis of mRAR α , mRAR β and mRAR γ poly(A)⁺ RNA from various mouse tissues (as indicated in a, lanes 1–14), and F9 EC cells (b, lanes 15–17) before (lane 15), and after 24 h (lane 16) and 48 h (lane 17) treatment with RA (3.3×10^{-7} M). Poly(A)⁺ RNA (4 μ g) was loaded in all lanes. The 28S and 18S rRNA standards were taken as being 4,712 (ref. 28) and 1,869 (ref. 29) bases long, respectively. a, mRAR α and mRAR γ sequences were detected with specific [³²P]-end labelled oligonucleotide probes. The entire mRAR β cDNA labelled with [³²P] by random priming was used to detect mRAR β RNA. Hybridizations were performed using the same blots, first hybridized with the mRAR α probe (exposure time, 7 days at -80°C ; two intensifying screens and Kodak XRO-5 film), then with the mRAR β probe and finally with the mRAR γ probe (exposure times, 4 days). The lower panel corresponds to a different blot hybridized only with a randomly primed entire mRAR γ cDNA probe (exposure time, 24 h). Actin RNA could be revealed with a cytoskeletal actin cDNA probe³⁰ in all cases except for pancreas and adrenal preparations suggesting RNA degradation (data not shown). b, Three blots were hybridized with randomly primed [³²P]-labelled entire mRAR α , mRAR β and mRAR γ cDNA probes (exposure time, 12 h). The filters were also probed with the actin cDNA probe (lower panel, ACT).

METHODS. RNA was extracted using the GdnSCN-CsCl procedure³¹. Poly(A)⁺ RNA³² was electrophoresed on 1% agarose–1.1 M formaldehyde gels³³ and transferred to nitrocellulose filters²⁴. Hybridisation was as described in the legend to Fig. 1, except that 50% formamide was used and hybridisation was at $42\text{--}45^\circ\text{C}$ ($37\text{--}40^\circ\text{C}$ for oligonucleotide probes) for 18 h. Filters were dehybridized by 5-min treatments in $0.05 \times \text{SSPE}$ at 90°C . Specific activity of all randomly-primed cDNA probes and labelled oligonucleotides was $\sim 10^9$ and 10^8 c.p.m. μg^{-1} DNA, respectively. The most stringent wash (15 min) was at 65°C in $0.1 \times \text{SSPE}$ plus 1.0% SDS/0.03% NaPPi for filters hybridized with randomly-primed cDNA probes and at 55°C in $1 \times \text{SSPE}$ plus 1.0% SDS/0.03% NaPPi with [³²P]-labelled oligonucleotide probes.

The expression of mRARs was investigated using specific oligonucleotide (mRAR α and mRAR γ) or randomly primed cDNA (mRAR β and mRAR γ) probes (Fig. 4a). Two mRAR α RNAs (~ 3.8 kilobase (kb) and 2.8 kb) were found in all mouse tissues including skin, and in 11.5-day old embryo (see legend to Fig. 4a for pancreas and adrenal). Compared with mRAR α RNA, the 3.4-kb mRAR β RNA was relatively more abundant in brain and in total 11.5-day embryo, and lower in skin and lung, than in other tissues. No 3.4-kb mRAR β RNA could be detected in spleen, intestine and testis, and an additional 1.9-kb species was found only in muscle (due to the use of different probes and exposure conditions, the mRAR α signal was amplified at least 20-fold relative to the mRAR α signal in Fig. 4a). By contrast, it is remarkable that mRAR γ RNA was detected at levels at least as high as those of mRAR α only in the skin of both 4-day-old and adult animals (upper and third row in Fig. 4a). Using a probe of higher specific activity, mRAR γ RNA was detectable in total 11.5-day embryo and in lung, and at trace levels in spleen (lower row in Fig. 4a). mRAR α and mRAR γ RNAs were also present in F9 EC cells which differentiate to endodermal-like cells upon exposure to RA²² (Fig. 4b). mRAR β RNA was induced by RA (at least 30-fold from densitometry), whereas no variation was seen for mRAR α RNA, and mRAR γ RNA decreased by two-fold. A similar induction of mRAR β RNA has been observed by L. Gudas *et al.* (personal communication). Whether this induction is transcriptional, as for hRAR β in a human cell line²³, and is mediated by mRAR α or mRAR γ or both, is unknown.

In summary, three RAR subtypes are expressed in the mouse, two of them being strikingly homologous to human RAR α and RAR β . That hRAR α and mRAR α , and hRAR β and mRAR β are more homologous to each other than either hRAR α and hRAR β , or mRAR α and mRAR β , strongly suggests that the three RAR subtypes exert specific functions perhaps by regulating the transcription of different genes at different times of development and in specific cells. In this respect, it is noteworthy that mRAR γ expression seems to be highly restricted to skin which is known to be an exquisite RA target in both normal and pathological states^{6–10}. Whether mRAR γ is specifically

expressed in keratinocytes remains to be seen. Finally, the numerous effects of RA on development and the presence of the three RARs in mouse embryo and differentiated F9 cells, raises the question as to whether they exhibit specific patterns of expression and function during embryogenesis. □

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A transcriptional repressor of *c-myc*

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IN murine plasmacytomas there is deregulated transcription of a translocated *c-myc* allele and undetectable transcription of the normal, unrearranged *c-myc* allele¹. Deregulated *c-myc* transcription probably contributes to the transformed phenotype of the tumour cells, whereas repression of the normal allele probably reflects the normal turn-off of *c-myc* in non-dividing plasma cells. We previously identified a plasmacytoma-specific protein which binds to the *c-myc* promoter region 290 base pairs 5' of the P1 transcription start site². This plasmacytoma repressor factor (myc-PRF; formerly myc-PCF) is not found in cell lines representing earlier B-cell stages during which *c-myc* is transcribed, so it could be a negative regulator of *c-myc* transcription in terminally differentiated B cells. Here we report that site-directed deletion of the binding site for this protein leads to a 30-fold increase in transcription of a stably transfected *c-myc* fusion construct in plasmacytoma cells but has no effect in L cells or 18-81 pre-B cells, which lack the protein. Myc-PRF interacts with another widely distributed protein, myc-CF1 (common factor 1), which binds nearby, and this association may be important in myc-PRF repression.

The binding sites for myc-PRF and myc-CF1, as identified by orthophenanthroline/copper (OP/Cu) chemical nuclease footprinting^{2,3} and methylation interference², are shown in Fig. 1 (also Fig. 3a). To test myc-PRF function, we used oligonucleotide-directed mutagenesis to delete 14 base pairs (bp) of the myc-PRF binding site (Fig. 1), thus eliminating all protein-DNA complexes resulting from myc-PRF binding *in vitro*, as assessed by a gel mobility shift assay (data not shown). Various portions of the *c-myc* promoter, with or without the deletion, were fused to the chloramphenicol acetyltransferase (*CAT*) gene (Fig. 1). Five pairs of wild-type and mutant promoter fusions were transiently transfected into the plasmacytoma line P3X63-Ag8 (P3X), and the *CAT* activity measured as an indicator of *c-myc* promoter activity. We find that only promoter regions with 3' ends extending 3' of exon 1 (*Bgl*II site, +568) give strong expression in our system (Fig. 1). Removal of a portion of exon

1 (429 bp *Hind*III-*Bgl*II) or addition of intron 1 sequences (313 bp *Bgl*II-*Bal*I or 696 bp *Bgl*II-*Bam*HI) at the 3' fusion site gives much reduced expression, which is at or below the level of detection of our 3-hour *CAT* assays (maximum linear reaction time). This is consistent with a positive element in exon 1 (ref. 4) and the role suggested for intron 1 as a block to transcription at the end of exon 1 which has been found in some systems³⁻⁷. Activity is doubled when an upstream region (716 bp *Bgl*II-*Sma*I) is deleted from the 1,709-bp *Bgl*II-*Bgl*II promoter/*CAT* fusion, consistent with a negative regulator ('dehancer') existing in that region⁸. Surprisingly, none of the promoters has increased activity when the myc-PRF site is deleted (Fig. 1).

The absence of a mutation effect might be explained if we could not detect a difference between wild-type and mutant promoters in transient transfections as a result of limited myc-PRF being titrated by the large excess of DNA templates in the transfected cells, in which case the fraction of templates repressed by myc-PRF would be undetectable. After *in vitro* competition experiments, we have also noted that myc-PRF is rare compared with other DNA-binding proteins in plasmacytoma nuclear extracts². We therefore transiently transfected 5–10 times less DNA (10 and 5 µg per 10⁷ cells) but activity was not reproducible, so we designed stable experiments in which transfected gene copy number was lower than in transient transfections, and in which sensitivity was improved because all cells contained the transfected construct.

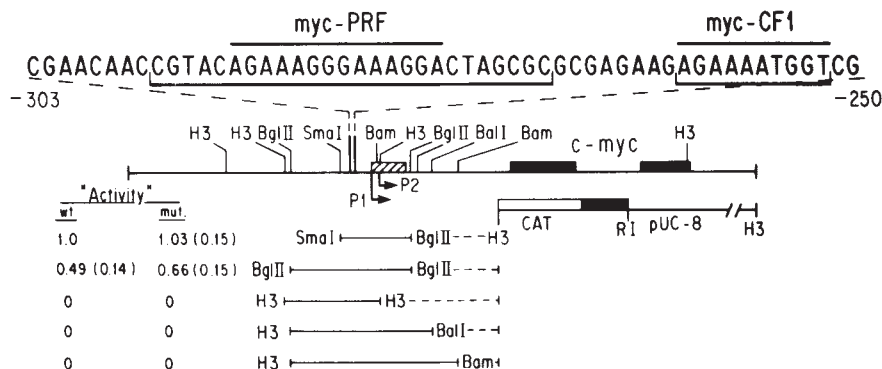
Co-transfection of pSV2NEO and the *Bgl*II-*Bgl*II myc/*CAT* construct into P3X cells was followed by selection for transformants with G418 (ref. 9). The *Bgl*II-*Bgl*II promoter was chosen because it is the largest promoter to show strong activity in transient transfections and it directs correctly initiated transcription⁸. From three experiments, we generated five separate pools containing 20–30 transformants each for both wild-type and myc-PRF site-deleted promoters (data not shown). The average copy number of intact transfected genes ranged from about 30 to 100 copies per cell in the different pools, as determined by Southern blotting (data not shown). *CAT* activity was low for extracts from the wild-type pools and increased by 31 times for the mutant pools (11.9 versus 0.39; Fig. 2 and Table 1). S1 nuclease analysis of RNA from two wild-type and two mutant pools showed that the increased transcription originated from the *c-myc* promoter, P2; no increased transcription was observed from other *c-myc* start sites (data not shown).

To prove that this increase in promoter activity when the myc-PRF site is deleted is not an artefact, we transfected mouse L cell fibroblasts and pre-B cell line 18-81, neither of which has myc-PRF binding activity (Fig. 3b). After stable transfection and selection with G418, Southern blotting showed that the four mutant and wild-type L cell pools contained equivalent copy numbers of transfected myc/*CAT* fusion genes, similar to those

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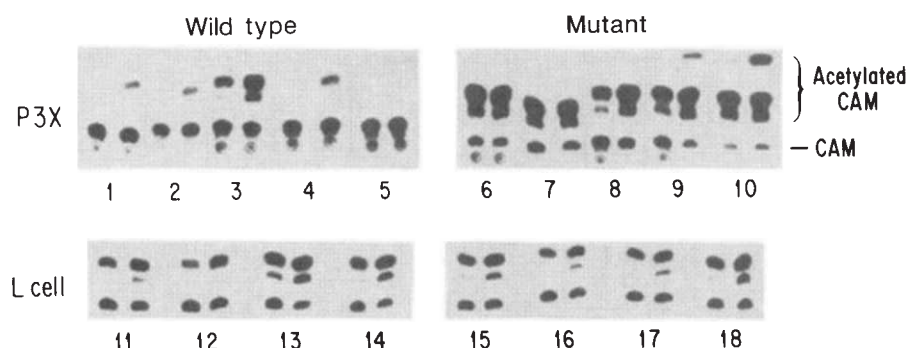
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FIG. 1 A map of the murine *c-myc* locus and activity of transiently transfected *c-myc*/*CAT* fusions. The *c-myc* gene is indicated by three boxes, the first non-coding exon is hatched (H3, *Hind*III; Bam, *Bam*HI). The binding sites of PRF and CF1 are indicated above the map. The overline indicates the conserved sequences and the brackets below indicate the OP/Cu footprint region. Below the map are indicated the constructions used in transfection experiments and the *CAT* activity shown upon transient transfection into P3X63-Ag8 cells. The indicated promoter fragments were end-filled (if necessary) and ligated to the *Hind*III-*Eco*R1 fragment of pSV2CAT in pUC-8 (ref. 16). During site-directed deletion of the PRF site, an addition of one 'T' residue resulted in the following sequence at the mutant PRF site: AACCGTA...T...ACTAGCGCGC. The mutation was then incorporated into the constructions shown. Transient transfection experiments used 50 µg per 10⁷ cells and calcium phosphate as before¹ using P3X; *CAT* was assayed in the usual way, with efficiency



controls by co-transfection of a β -galactosidase-expressing construct¹. The data for 4 or more experiments are normalized to the *Sma*-*Bgl* wild-type promoter, with the standard deviation in parentheses. The zero indicates unreliably low activity.

FIG. 2 Autoradiographs of CAT activity assays of stable *BglIII-BglIII*/CAT transformants. Stable transformants of P3X were made by calcium phosphate transfection of 2 μ g pSV2NEO and 20 μ g each construct. Colonies arising after 2–3 weeks of G418 selection in 24-well plates were pooled in groups of 20 or more and assayed after 3–4 passages in selective media. Autoradiographs of thin layer chromatography plates show the separated products of the CAT assay. Two time points for each extract are shown (10 and 30 min). The assays of five extracts derived from P3X cells containing wild-type (1–5) or mutant *myc*/CAT fusions (6–10) are shown above; the assays of L-cell stable transformants with wild-type (11–14) and mutant *BglIII-BglIII* promoters (15–18) are below. Increased acetylated chloramphenicol (CAM) (two or three upper spots) versus lowest spot (unacetylated)



indicates increased CAT expression in the cells. The extract from $\sim 5 \times 10^6$ P3X cells and 5×10^5 L cells was used in the assays. Quantitation is shown in Table 1.

in P3X cells (data not shown). There was no significant difference in CAT activity between the wild-type and mutant pools (Fig. 2; Table 1), but CAT expression in L cells was ~ 7 -fold higher than in P3X cells containing the *myc*-PRF site deletion, indicating that additional sequences or post-transcriptional processes could be altering *c-myc*/CAT expression in these cells. Using five wild-type and two mutant stable clonal cell lines in pre-B cells (18–81), we found variation among different clones, but clonal lines containing mutant *myc*/CAT were no higher than lines containing wild type (46.7 and 103, versus 67.6, 365, 10.2, 7.1 and 22 picomol chloramphenicol converted per hour per 10^7 cells). Deletion of the *myc*-PRF site therefore has no detectable effect in cells lacking the factor.

So, deletion of the *myc*-PRF binding site releases the *c-myc* promoter and allows a 30-fold increase in expression in plasmacytoma cells containing *myc*-PRF, but in L cells or pre-B cells which cannot bind *myc*-PRF, there is no effect. We conclude that the protein that binds at the *myc*-PRF site represses *c-myc* transcription and is responsible, at least in part, for the repression of the normal *c-myc* gene in plasmacytomas. We infer from partial purification (see below) that only one protein is binding at this site, but more than one could be involved.

We investigated the multiple *myc*-PRF complexes seen in gel mobility shift experiments and the cell type distribution of *myc*-PRF and *myc*-CF1. In plasmacytomas, the 'C', 'D' and 'E' complexes detectable in a mobility shift assay using a probe from the *c-myc* promoter (–421–208 bp) seem to arise from binding at the PRF site only, on the basis of our OP/Cu footprinting experiments². These multiple complexes could result from proteolytic degradation of *myc*-PRF, post-translationally modification of *myc*-PRF or interaction of *myc*-PRF with other proteins. The 'B' complex seems to be attributable to a single factor, *myc*-CF1, which protects the sequence AGAAATGGT at 10 bp 3' of the *myc*-PRF site² (Fig. 3a). From footprinting experiments, the 'A' complex results from binding of *myc*-PRF and *myc*-CF1 (ref. 2; and see Fig. 3a). We do not understand yet why the relative amounts of the A, C, D and E complexes should vary in different preparations and cell lines.

We then partially purified *myc*-PRF using DEAE-Sepharcel, heparin-Sepharose, FPLC mono-Q anion exchange, and an oligonucleotide affinity column (data not shown). The C, D and E complexes co-purified, including over two final passes on the affinity column. The A complex was lost at the mono-Q step where *myc*-PRF and *myc*-CF1 activities were separated. During the partial purification and after the final fractionation step, the activity of the C and D complexes decreased and increased for the E complex, so the larger complexes could be undergoing conversion to a smaller, higher-mobility form. We used the purified material in an OP/Cu footprinting experiment of the E complex, which gave the same footprint as the crude or heparin-Sepharose-fractionated extracts² (Fig. 3a). OP/Cu foot-

printing of the B complex using an EL4 T-cell extract devoid of other binding activities for this probe also showed the same footprint as extracts from pre-B and plasmacytoma cells² (Fig. 3a).

Figure 3b shows binding of *myc*-PRF and *myc*-CF1 in different tissues. *Myc*-CF1 (complex B) is found in all the cell lines and tissues we tested, except for mouse brain (Fig. 3b and data not shown), whereas large amounts of complexes A, C, D and E (dependent on *myc*-PRF binding) are found only in plasmacytomas (M603, P3X, S107 in Fig. 3, and J558L in ref. 2). Some complex A is also found in the Ly1+ B cell line, 1414, a subclone of 750-13 which contains an amplified *c-myc* gene¹⁰. We confirmed specific binding to the PRF site in these complexes by competition with a double-stranded 25-bp oligodeoxynucleotide containing the PRF binding site (Fig. 3c, and data not shown). The other complexes in brain, liver and kidney extracts (Fig. 3b) do not compete with the PRF-site oligonucleotide (data not shown). We also tested nuclear extracts from human cell lines with a human DNA probe containing the homologous PRF/CF1 site sequences (*RsaI-SmaI* fragment) and found that none of the cell lines, including the Burkitt lymphomas Daudi and Manca, contained *myc*-PRF, although *myc*-CF1 was present in abundance (Fig. 3 legend; data not shown). Furthermore, murine *myc*-PRF in M603 extract did not bind the human DNA probe, although murine *myc*-CF1 bound the human DNA well (data not shown).

TABLE 1 Stable transfection of *myc*/CAT fusion constructs into P3X and L cells

P3X plasmacytoma pools			
PRF mutant		Wild type	
Pool	Activity	Pool	Activity
2A	9.65	1A	0.19
2B	5.65	1B	0.56
4A	5.0	3A	1.1
6A	10.9	3B	0.1
6B	28.5	5C	0.013
Average	11.9		0.39
L-cell fibroblast pools			
2AL	68.6	1AL	51.5
2BL	58.2	1BL	37.5
4AL	78.0	3AL	127
4BL	134	3BL	153
Average	84.7		92.0

Activity represents picomoles chloramphenicol acetylated per hour per 10^7 cells. The P3X mutant pools' activity of 11.9 ($\pm$ s.d. 9.6) would be 7.8 ($\pm$ 2.7) if the one high value of 28.5 (pool 6B) were omitted. This is still about 20-fold higher than the wild type P3X pools value of 0.39 ($\pm$ 0.45).

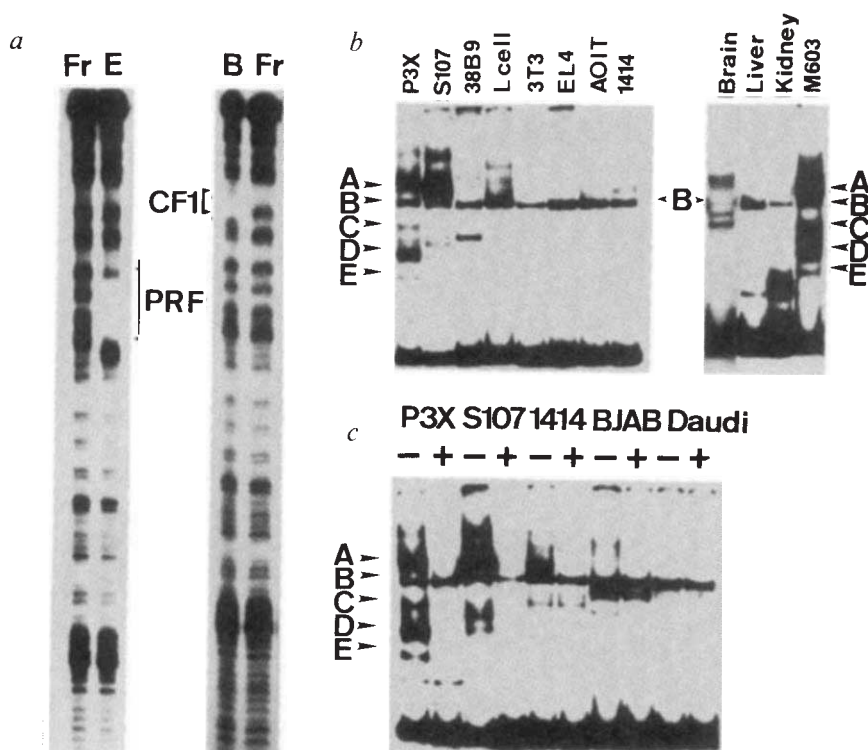


FIG. 3 *a*, OP/Cu footprinting of DNA-protein complexes containing PRF and CF1. 'Fr', DNA not bound by protein; 'E' or 'B', the specific DNA-protein complex. On the left, partially purified PRF (using two passes of affinity chromatography) was used. On the right, the footprint of the B complex derived from CF1 factor in a crude nuclear extract of EL4 T cells is shown. *b*, Autoradiographs of mobility shift experiments with various extracts are shown. A 213-bp probe (**Sma*-*Hpa*II) with the PRF and CF1 sites from the murine *c-myc* locus was used with nuclear extracts in mobility shift experiments as described previously². 38B9 is an Abelson virus transformed pre-B cell which shows the 'B' and a lower 'F' complex seen in other early B cell lines². The other cell lines are: L and 3T3, fibroblasts; EL4 and AOT, T-cell lines; 1414, a B-cell line (see text); organs from Cx2D F1 mice; BJAB, B-cell lymphoma; Daudi, a Burkitt lymphoma. The following cell lines (not shown) have the B complex resulting from CF1: COL0320, HL60 (with and without 2 h, 2 days and 6 days of 1.25% dimethylsulphoxide treatment) and Manca Burkitt cells, which also contain the 'F' complex of early B cells. *c*, Double-stranded PRF oligodeoxynucleotide competition experiments. Indicated are the lanes with (+) and without (-) 2.5 ng of the 25-bp competitor

CGCGTACAGAAAGGGAAGGACTAG
ATGTCCTTCCCTTCTCTGATCGCGC

These results show that myc-CF1 is present in many cell types and that myc-PRF seems to be restricted to terminally differentiated B cells, represented by plasmacytomas. The normal *c-myc* allele in plasmacytomas is repressed in transcription¹. The absence of myc-PRF in the other immortalized cell lines correlates with their earlier developmental stage and detectable transcription of normal *c-myc* in most cases. The Burkitt lines tested probably represent earlier stages of B-cell development¹¹ although they do not transcribe normal *c-myc*. Lack of myc-PRF in these lines suggests that alternative mechanisms are repressing *c-myc* in these cells¹²⁻¹⁴. Our preliminary results with the normal mouse brain, kidney and liver indicate that other differentiated tissues may not contain myc-PRF, but it is possible that we did not detect a low activity of myc-PRF or of a small fraction of cells in the tissues.

We used partially purified myc-PRF free of myc-CF1 to study the association of myc-PRF and myc-CF1 (Fig. 4, lane 2), which bind near to one another (Fig. 1). Increasing amounts of myc-PRF added to crude nuclear extract from the T-cell line EL4, containing myc-CF1 but no myc-PRF, gave complex A formation and eventual elimination of complex B (Fig. 4). A similar result was obtained with myc-PRF and 18-81 pre-B cell extract (data not shown). Hence, formation of complex A requires at least myc-PRF and myc-CF1. The lack of myc-PRF binding in EL4 extracts is probably not due to an inhibitor, although an inhibitor could be titrated out by excess myc-PRF (Fig. 4). We cannot yet exclude a contribution from other non-DNA-binding factors to the formation of complex A.

To investigate whether the association of myc-PRF and myc-CF1 alters their binding to DNA, we compared the half lives of the complexes 'A' (PRF+CF1), 'B' (CF1) and 'C' (PRF) in off-rate experiments. After the binding of both proteins in a P3X nuclear extract had reached equilibrium, a large excess of unlabelled probe was added and aliquots loaded onto a native polyacrylamide gel at timed intervals. Half-lives were measured by densitometry of autoradiographs in five separate experiments, one of which is shown in Fig. 4. Half-lives were: complex B <30 s, complex C, 17.5+/-5.1 min, and complex A, 21.7+/-3.0 min. This demonstrates that PRF and CF1 interact to significantly affect binding of CF1, and possibly of PRF as well. The function of CF1 is unknown, but its wide tissue distribution indicates that it may be a positive *c-myc* transcription factor. Interaction between PRF and CF1 could be a mechanism for repression by myc-PRF.

Regulation of *c-myc* transcription is very complex, as evidenced by the large number of regulatory regions and the large conserved promoter region¹⁵. Here we have shown that myc-PRF strongly represses *c-myc* transcription in fusion constructs transfected into murine plasmacytomas and so could be important in the repression of *c-myc* in non-dividing plasma cells, which may occur through interaction with a ubiquitous *c-myc* promoter-binding protein, myc-CF1. The role of the CF1-PRF interaction and how myc-PRF repression is integrated with the numerous other *c-myc* regulatory mechanisms remain to be determined. The loss of the upstream regulatory region, includ-

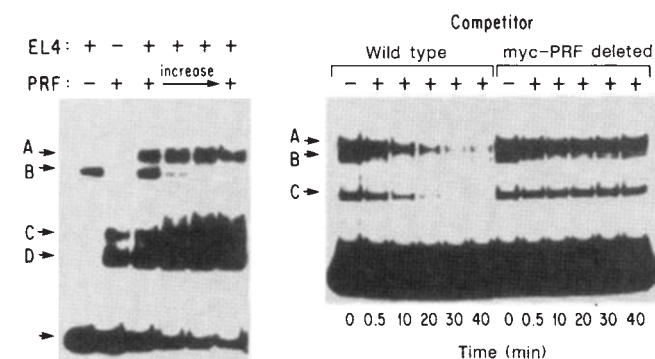


FIG. 4 Left panel: mixing experiment. One-pass oligo-column purified PRF (complexes C, D; lane 2) is added to EL4 extract (B; lane 1) and assayed by gel mobility shift to generate complex A (lane 3). Increasing PRF (0.25, 0.5, 1.0, 1.5 μ l purified PRF) is added to lanes 3-6. Right panel: half-life of complexes measured by gel-mobility shift. P3X nuclear extract was incubated with the *Sma*-*Hpa*II probe in a standard binding reaction for 20 min at 22° and then 200 ng (200-500-fold excess) of unlabelled PRF site wild-type or mutant competitor DNA (*Ava*I fragment containing the PRF/CF1 sites) was added. After competitor addition, samples were loaded onto a 7% native polyacrylamide gel at the times indicated.

ing the PRF site, following *c-myc* translocation in plasmacytomas is consistent with a role for *myc*-PRF in repression². However, no specific natural mutations of the PRF site have been reported. That *myc*-PRF represses transcription from the *c-myc* promoter is significant to the understanding of both complex *c-myc* regulation and how terminally differentiated B cells can prevent cell division. □

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Serpin-resistant mutants of human tissue-type plasminogen activator

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TISSUE-type plasminogen activator (t-PA) converts the inactive zymogen, plasminogen, into the powerful protease, plasmin, which then degrades the fibrin meshwork of thrombi^{1–3}. To prevent systemic activation of plasminogen, plasma contains several inhibitors of t-PA, the most important of which is plasminogen activator inhibitor-1 (PAI-1), a member of the serpin superfamily^{4–6}. As the ability to produce serpin-resistant variants of t-PA could increase the potential of this enzyme as a thrombolytic agent, we have used the known three-dimensional structure of the complex between trypsin and bovine pancreatic trypsin inhibitor (BPTI) to model the interactions between the active site of human t-PA and PAI-1. On the basis of this model we then altered by site-directed mutagenesis those amino acids of t-PA predicted to make contact with PAI-1 but not with the substrate plasminogen. We report here that although the resulting mutants have enzymatic properties similar to those of wild-type t-PA, they display significant resistance to inhibition by PAI-1. For example, following incubation with an amount of the serpin that completely inhibits the wild-type enzyme, one variant retains 95% of its initial activity. This mutant is also resistant to inhibition by the complex mixture of serpins present in human plasma.

PAI-1 (relative molecular mass (M_r) 50,000) has been implicated as the cause of reduced fibrinolytic capacity of plasma from survivors of myocardial infarctions^{7,8}. The second-order rate constant for association of PAI-1 with t-PA is extremely high⁹ ($\approx 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and accounts for the initial, 'fast-phase'

inhibition of t-PA by human plasma¹⁰. In addition to PAI-1, other protease inhibitors such as C1 esterase inhibitor and alpha-2 antiplasmin are present in abundant quantities in plasma¹¹. Although their association constants for t-PA are orders of magnitude lower than that of PAI-1^{9,12}, these serpins nevertheless bind to infused t-PA⁸ and may attenuate the beneficial pharmacological properties of the enzyme.

The high homology between the catalytic domain of t-PA and that of the other members of the chymotrypsin family of serine proteases^{13,14} together with the structural similarity among serine protease-inhibitor complexes formed between members of different families of proteases and inhibitors^{15,16}, prompted us to consider the possibility that the known structure of the complex between trypsin and BPTI^{17,18} might serve as a model for the interaction between t-PA and PAI-1. Contacts between trypsin and BPTI, other than those in the principal recognition site, involve two separate regions of the trypsin polypeptide chain

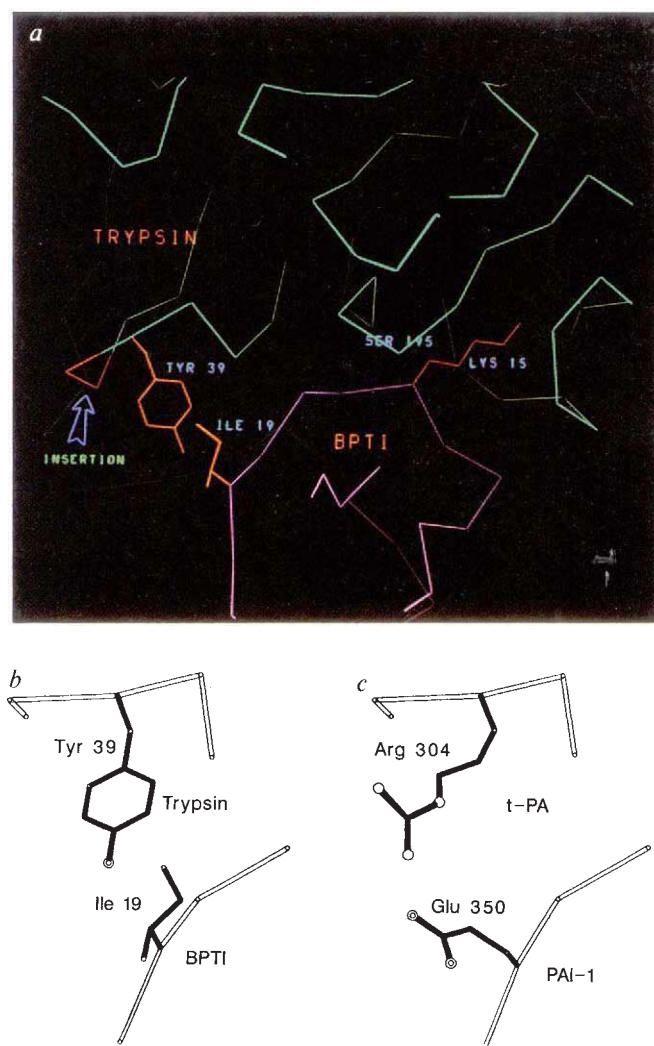


FIG. 1 A model of the interaction between t-PA and PAI-1. *a*, The region of interaction between bovine pancreatic trypsin and BPTI¹⁷ (Protein Databank file 2ptc. dat), displayed in Insight³⁴. The α -carbon backbone of trypsin is shown in blue-green and that of BPTI is shown in magenta. The sidechains of Tyr 39 of trypsin and Ile 19 of BPTI, which are in van der Waals contact, are shown in red. Also shown in red is the sidechain of Lys 15 of BPTI, which lies in the P1 recognition site of trypsin. The position of the insertion Lys(296)-His-Arg-Ser-Pro-Gly(302) in t-PA is indicated (residues not modelled). *b*, Close-up view of the van der Waals contact between Tyr 39 of trypsin and Ile 19 of BPTI. *c*, A model of the possible interaction between Arg 304 of t-PA and Glu 350 of PAI-1, based on the assumption that the active-site loop of PAI-1 takes up a conformation similar to the active-site loop of BPTI.

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(residues 37–41 and 214–217; see Table 1). The amino-acid sequence Ser(210)–Trp–Gly–Ser(213) is highly conserved among members of the chymotrypsin family of serine proteases, suggesting that these residues play an essential structural or catalytic role. By contrast, the region around amino-acid residues 36–41 of trypsin is more variable and forms part of the surface that interacts with the inhibitor. The amino-acid sequence of t-PA in this region differs from that of trypsin in two major respects (see Table 1). First, Tyr 39 of trypsin has been replaced with an arginine residue (Arg 304). Modelling based on the assumption that the interaction between t-PA and PAI-1 mimics that of trypsin and BPTI (Fig. 1) suggests that Arg 304 can form a salt bridge with Glu 350 of PAI-1. This glutamic acid residue is equivalent in position to Ile 19 of BPTI which forms a van der Waals contact with Tyr 39 of trypsin^{17,18}. Second, t-PA carries an additional stretch of seven amino acids (Lys(296)–His–Arg–Arg–Ser–Pro–Gly(302)) inserted adjacent to the predicted contact between t-PA (Arg 304) and PAI-1 (Glu 350) and near the entrance to the catalytic site (see Table 1 and Fig. 1). Interestingly, four of these seven amino acids are positively-charged, whereas the predicted complementary region

of PAI-1 (Glu(350)–Glu–Ile–Ile–Met–Asp(355)) contains three negatively-charged residues. It seemed possible that electrostatic interactions between these regions might play an important role in the formation or stabilization of the complex between t-PA and PAI-1. By contrast, such interactions could not occur when t-PA interacts with its substrate, plasminogen, which has no negatively-charged residues in the equivalent region¹⁹.

To test the hypothesis outlined above, namely that residues Lys(296)–His–Arg–Arg–Ser–Pro–Gly(302) and Arg 304 of t-PA interact with PAI-1 but not with plasminogen, oligonucleotide-

TABLE 1 Sequence alignment of human t-PA with trypsin

	16	37	39	41	60
Trypsin	IVGGYTCGAN	TVPYQVSLNS	GYH	FCGGSLINSQ	WVWSAAHCYK
t-PA	IKGGFLADIA	SHPWQAIFA	KHRRSPGGRF	LCGGILISSC	WLSAAHCFQ
	276	296	304		325
	61				105
Trypsin	S.....GIQV	RLGEDNINVV	EG.NEQFISA	SKSIVHPSYN	SNTLNNDIML
t-PA	ERFFPHHLTV	ILGR.TYRVV	PGEEEQKFV	EKYIVHKEFD	DDTYNDIAL
	326				374
	106				150
Trypsin	IKLKA....	ASLNSRVAS	ISLPT..SCA	SAGTQCLISG	WGNTKSSGTS
t-PA	LQLKSDSSRC	AQESSV.VRT	VCLPPADLQL	PDWTECELSG	YKGHEALSPF
	375				423
	151				190
Trypsin	YPDVLKCLKA	PILSDSSCKS	A..YPGQITS	NMFCAGYL.E	GG.....KDS
t-PA	YSERLKEAHV	RLYPSSRCTS	QHLLNRTVTD	NMLCAGDTRS	GGPQANLHDA
	424		214		473
	191				240
Trypsin	CQGDGGGPPV	CS....GKLQ	GIVSWGSGCA	QKNKEGVYTK	VCNVVSWIKQ
t-PA	CQGDGGGPLV	CLNDGRTMLV	GIISWGLGCG	QKDVFGVYTK	VTNYLDWIRD
	474				523
	245				
Trypsin	TIASN				
t-PA	NMRP				
	527				

A comparison of the amino-acid sequences of the light chain of human t-PA with trypsin. This alignment shows the high degree of conservation between the two serine proteases^{13,14}. The numbers for trypsin refer to the numbering system used in the PDB 3pt entry in the Protein Databank. The numbers for t-PA refer to the sequence of amino acids in the mature t-PA molecule²⁵. t-PA has five small insertions of amino acids that are absent from trypsin but map as loops on the surface of the molecule¹⁴. One of these insertions is adjacent to a region which in trypsin (residues 39–41) forms van der Waals contacts with BPTI^{17,18}. This region of t-PA (residues 296–304) was chosen for site-directed mutagenesis. The synthetic oligonucleotides used to generate the following mutants were:

t-PA(del 296–302)	5'-GCCATCTTTGCCGAGCGGTTCTG-3'
t-PA(Arg→Ser 304)	3'-GCCCGGAGAGTCGTTCTGTGC-3'
t-PA(Arg→Glu 304)	5'-GCCCGGAGAGGAGTCTGTGC-3'

Mutations were introduced as described by Kunkel²⁰ into the 472-base pair (bp) central *EcoRI* fragment of wild-type t-PA cDNA²⁵ that had been cloned into M13mp18 (ref. 31). The resulting DNA was used to transfect *Escherichia coli* strain TG-1 and single-stranded DNA was prepared from several plaques. The 472-bp tracts of t-PA cDNA in several of these single-stranded DNA templates were completely sequenced³² to confirm the presence of the desired mutations. The double-stranded replicative form of the DNAs of proven mutants was then isolated and the mutant 472-bp fragments were isolated by digestion with *EcoRI* and electrophoresis through agarose gels³³. These fragments were then used to replace the central 472-bp *EcoRI* fragment of wild-type t-PA cDNA²⁵ cloned in pSVT7(RI⁻), which is a version of the mammalian expression vector pSVT7 (refs 21 and 22) that lacks the *EcoRI* site in the polylinker. The recombinant methods used are described by Sambrook *et al.*³³.

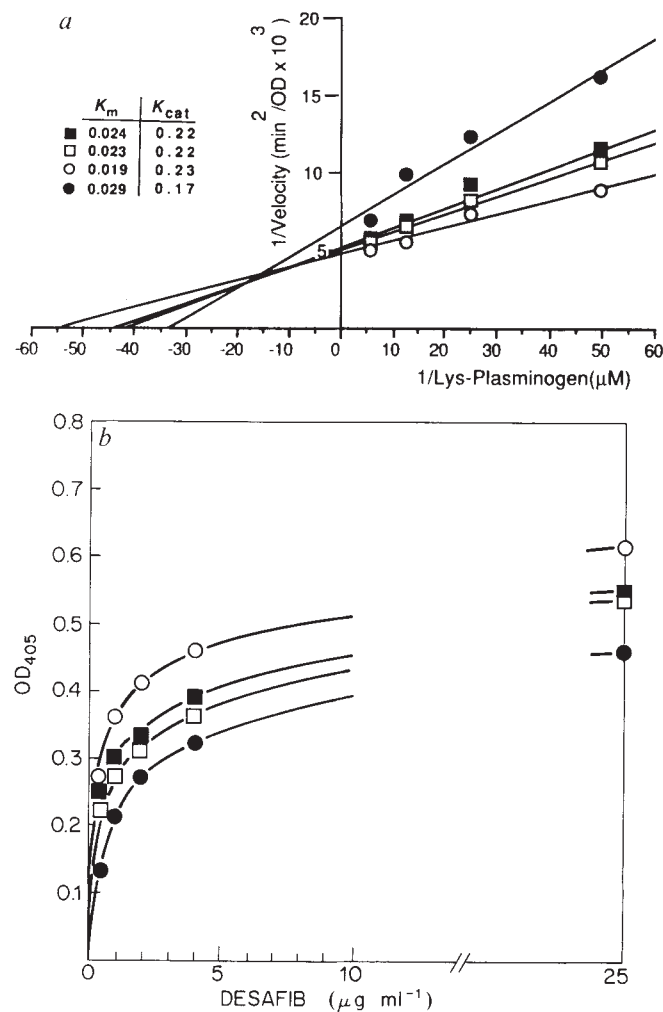


FIG. 2 Comparison of the activities of wild-type and mutant t-PAs. cDNAs encoding wild-type and mutant t-PAs were cloned into the mammalian expression vector pSVT7(RI⁻). COS-1 cells²⁴ were transfected with 1 μg of the appropriate plasmid DNAs essentially as described²⁵. 12 h after the chloroquine boost, the medium (DMEM containing 10% fetal bovine serum) was replaced with medium lacking serum and incubation was continued for a further 60 h before the conditioned medium was collected. The concentration of t-PA protein in the medium, as determined by solid-phase radioimmunoassay²¹, was approximately 500 ng ml⁻¹. t-PA activity was measured by a standard indirect chromogenic assay described previously^{26,27} using reaction mixtures (100 μl) that contained 150 pg of the t-PA to be tested, 0.4 mM Spectrozyme PL (American Diagnostica Inc.), 25 μg ml⁻¹ DESAFIB (American Diagnostica Inc.) and varying concentrations of Lys-plasminogen (American Diagnostica Inc.). *a*, Lineweaver-Burke plots of the activity of wild-type and mutant t-PA enzymes. The units for the K_m and K_{cat} values shown in the insert are μM and s⁻¹, respectively. *b*, Stimulation of the wild-type and mutant enzymes by fibrin monomers. Antigenically equal amounts of the wild-type and mutant t-PA enzymes were assayed by the standard indirect chromogenic assay^{26,27} using reaction mixtures (100 μl) that contained 150 pg of the t-PA to be tested, 0.4 mM Spectrozyme PL, 0.16 μM Lys-plasminogen and varying concentrations (0–25 μg ml⁻¹) of the soluble fibrin monomer, DESAFIB. ■ wild-type t-PA; □ t-PA(Arg→Ser 304); ● t-PA(del 296–302).

directed mutagenesis²⁰ was used to produce three mutant forms of the serine protease (Table 1). Of these t-PA(del 296–302) lacks the seven amino-acid insertion, whereas mutants t-PA(Arg → Ser 304) and t-PA(Arg → Glu 304) have Arg 304 substituted by residues that cannot form a salt bridge with Glu 350 of PAI-1. The complementary DNAs encoding wild-type t-PA²¹ and the three mutant enzymes (see legend to Table 1) were cloned into a plasmid vector, pSVT7(RI⁻), that contains the controlling elements derived from the early gene of SV40 (refs 22 and 23) and expressed in COS-1 cells²⁴ as described previously²⁵. The wild-type and mutant t-PAs secreted into serum-free medium from 16–76 hours after transfection were either assayed without purification, or were immunopurified²⁶ before measurement of enzymatic activity in a standard indirect chromogenic assay^{26,27}. All three mutants are enzymatically active and their specific activities are very similar to that of the recombinant wild-type t-PA or of standardized, commercially-available preparations of t-PA purified from the Bowes line of human melanoma cells. Lineweaver-Burke plots of the kinetic data obtained when wild-type t-PA and the three mutants were assayed in the presence of saturating concentrations of the fibrin monomer DESAFIB (25 $\mu\text{g ml}^{-1}$) and different concentrations of the substrate Lys-plasminogen are shown in Fig. 2. The values of K_m and K_{cat} for wild-type t-PA and the three mutants (Fig. 2a inset) are similar to one another and to values previously published by our laboratory^{26,27} and others^{28,29}. The results shown in Fig. 2b demonstrate that the mutants respond to the positive effector, DESAFIB, in a manner similar to the wild-type enzyme. The maximal stimulation was 20–40-fold, in agreement with our previous results^{26,27} and those of others using the

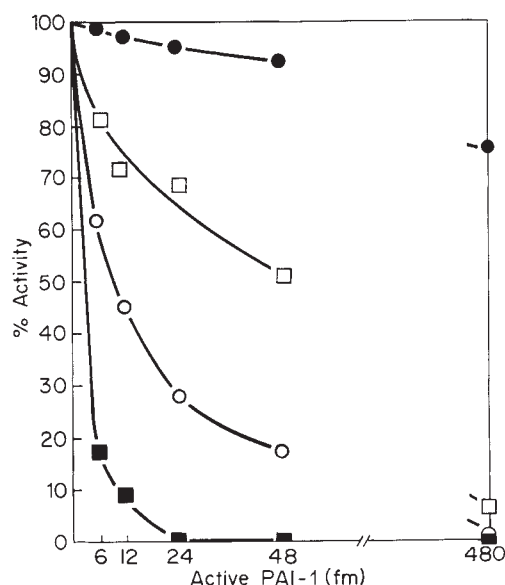


FIG. 3 Effect of PAI-1 on the activity of wild-type and mutant t-PA enzymes. Recombinant human PAI-1 was partially purified⁹ from the supernatant medium of simian CV-1 cells that had been infected with a late-replacement vector of SV40 carrying a cDNA coding for human PAI-1 (Gerard, R. D. and Meidell, R. S., unpublished experiments). The preparation used to obtain the data shown contained 48 nM active inhibitor. Approximately equal amounts of mutant and wild-type t-PAs (3.8 fmoles diluted to 25 μl with DMEM) were incubated with varying amounts of PAI-1 (0–480 fmoles of active inhibitor diluted to 10 μl in assay buffer) for 20 min at room temperature. The residual activity of the enzymes was then determined using the indirect chromogenic assay^{26,27} in the presence of 0.4 mM Spectrozyme PL, 0.16 μM Lys-plasminogen and 25 $\mu\text{g ml}^{-1}$ of the soluble fibrin monomer, DESAFIB. ■ wild-type t-PA; ○ t-PA(Arg → Ser 304); □ t-PA(Arg → Glu 304); ● t-PA(del 296–302). The results shown were obtained using unfractionated mutant and wild-type enzymes from conditioned medium. Identical results, however, have been obtained using enzymes purified by immunoaffinity chromatography²⁶ (R. Bassel-Duby, unpublished data). The wild-type and mutant proteins remained in the single-chain form throughout purification and the subsequent incubation with PAI-1.

DESAFIB preparation³⁰. In each case, half-maximal stimulation occurred when DESAFIB was present at a concentration of approximately 1 $\mu\text{g ml}^{-1}$. These data show that deletion of residues 296–302 of t-PA or substitution of Ser or Glu for Arg 304 have little effect on the abilities of t-PA to activate plasminogen and to be stimulated by soluble fibrin fragments.

To test whether these variant t-PAs exhibit altered interaction with PAI-1, the mutant and wild-type forms of the enzyme were preincubated with various concentrations of partially-purified recombinant PAI-1 and the residual enzymatic activity was then measured using the indirect chromogenic assay. The results, presented in Fig. 3, show that all three mutants behave quite differently from wild-type t-PA. Under conditions where wild-type t-PA is completely inhibited by PAI-1, the deletion mutant t-PA(del 296–302) retains $\approx 95\%$ of its activity. Only at the highest concentrations of PAI-1 does this mutant display any significant diminution of enzymatic activity. At such high concentrations, when PAI-1 is present in ≈ 125 -fold molar excess over t-PA and at a concentration 20-fold less than that of lys-plasminogen, it is possible that PAI-1 acts as a standard competitive inhibitor rather than as a suicide substrate. The two substitution mutants also are resistant to the inhibitor, although to different extents. Mutants containing Ser or Glu instead of Arg 304 require ≈ 4 and 25 times more PAI-1 for half-maximal inhibition of enzyme activity than does wild-type t-PA (Fig. 3).

To determine whether t-PA(del 296–302) also exhibits resistance to the complex mixture of serine protease inhibitors present in human plasma⁴, a 100-fold dilution of human plasma was substituted for the partially-purified recombinant PAI-1 in the protocol described in the legend to Fig. 3. Under these conditions $\approx 70\%$ of the activity of the wild-type enzyme was inhibited whereas the activity of t-PA(del 296–302) was unaffected. In separate experiments, wild-type t-PA and the deletion mutant were incubated with undiluted human plasma and then the mixtures were acidified to pH 5 and centrifuged for 5 min at 12,000 $\times g$. The clarified supernatants were diluted and assayed for residual t-PA activity, which totalled more than 90% for t-PA(del 296–302) and 20% or less for the wild-type enzyme.

Our data strongly suggest that residues 296–302 and 304 are not involved in catalytic functions of t-PA, but play an important role in the interaction of the enzyme with its cognate serpin, PAI-1, and with other inhibitors present in serum. Using the structure of trypsin as a model, these residues are predicted to map on the lip of the active site of the serine protease, some distance from the catalytic triad. We therefore conclude that the area of contact between t-PA and PAI-1 is more extensive than the interaction between the enzyme and its true substrate plasminogen. Although a more detailed mutational analysis of residues 296–304 and other regions of the t-PA light chain is required to define more precisely the area of contact that is unique to PAI-1, our results open the way to explore the specificity of similar interactions between other medically important serine proteases and their cognate serpins, such as elastase and α_1 -antitrypsin. In addition, the use of variants of t-PA that are resistant to inhibition by serpins offers a promising approach to maintain the enzyme in an active form in the circulation of patients treated for myocardial infarction. □

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The highly concentrated liquid-crystalline phase of DNA is columnar hexagonal

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THE DNA molecule is extremely compacted in bacteria, in cell nuclei, sperm heads and virus capsids. These interactions between DNA molecules are important to our understanding of chromatin condensation. DNA forms multiple liquid-crystalline phases whose nature depends on the polymer concentration¹⁻¹², and it has been suggested that the highly concentrated phase of 50-nm DNA molecules is two-dimensionally ordered and smectic-like¹³. We rule out this smectic hypothesis and demonstrate by polarizing microscopy, electron microscopy and X-ray diffraction that this phase is characterized by a columnar longitudinal order and a hexagonal lateral order, with intermolecular distances ranging from 2.8 to 4.0 nm depending on the DNA concentration.

We prepared large amounts of 50-nm DNA molecules¹⁴ and

obtained the liquid crystalline phases using distilled water, 0.1 M NaCl and 0.25 M ammonium acetate as solvents without any visible modification of texture in the optical microscope. The first germs of the 'highly concentrated' phase are either diamond-shaped or disc-shaped (Fig. 1*a, b*). They grow and merge to form a homogeneous phase with textures that are undulating, fan-shaped, and striated fan-shaped (Fig. 1*c, d, e*). The same textures were seen in the homologous phase consisting of long DNA molecules¹¹, and are characteristic of lamellar systems. Smectic and columnar hexagonal phases can both be considered as lamellar structures (Fig. 2*a, b*). In a smectic phase, molecules are normal (or oblique) to the layer plane and the thickness of each layer is close to the length of the molecule. In a columnar hexagonal phase, the three equivalent directions of the hexagonal network define three series of parallel layers; molecules are lying in the plane of these layers whose thickness is then close to the interhelix spacing (Fig. 2*b*). To rule out one of the two models, a simple experiment was carried out. A suitable situation is found when the layers are folding around a disclination line that is normal to the preparation plane (Fig. 2*c, d*), when the molecules that are normal to the layers in a smectic phase will now be aligned parallel to the lines radiating from the disclination line. These molecules will be oppositely orientated in a columnar phase, that is, normal to the lines radiating from the disclination line. Taking into account the intrinsic negative birefringence of the DNA molecules, we used a quartz first-order retardation plate (inserted at 45° between the crossed polarizers) to determine the molecular orientation in such domains. Molecules are normal to the radii of these

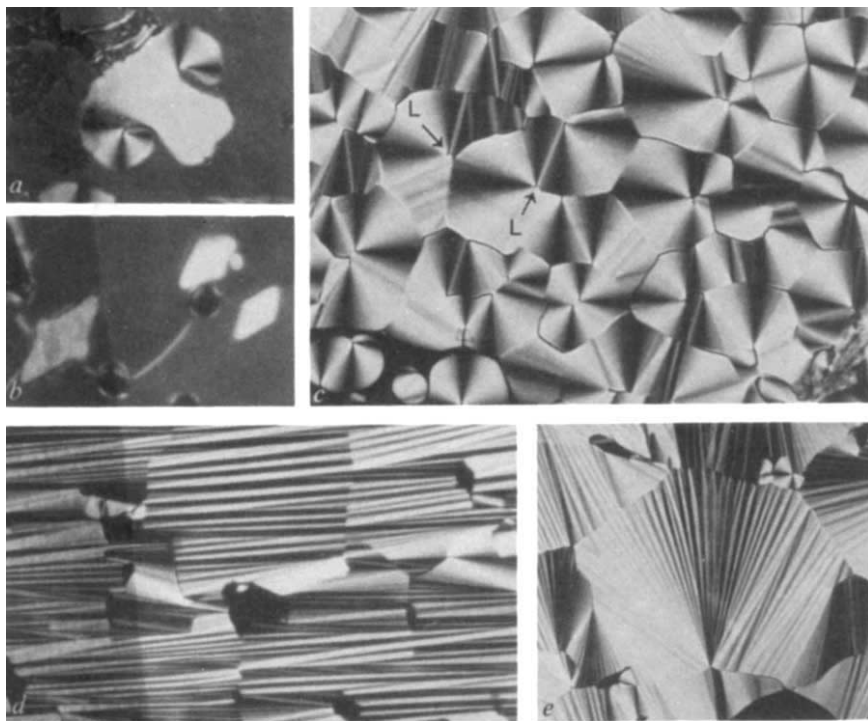


FIG. 1. Highly concentrated liquid-crystalline phase of DNA observed by polarized-light microscopy. A solution of 200 mg ml⁻¹ DNA was deposited between slide and coverslip. The concentration was progressively increased by slow evaporation of the solvent and monitored between crossed polarizers in a Nikon Optiphot X Pol microscope. *a* and *b*, Highly birefringent germs of the hexagonal phase grow in the nearly extinguished planar cholesteric phase. They can be either disc (*a*) or diamond-shaped (*b*). *c, d, e*, The hexagonal germs coalesce to form a homogeneous phase. Most of the domains articulate around disclination lines (L). Magnification 135× in *a, e*; 180× in *b, d*; 320× in *c*. DNA fragments of about 146 base pairs (~50 nm) were prepared by digestion of calf-thymus chromatin with micrococcal nuclease¹⁴. The textures observed in polarizing microscopy were shown to be independent of the saline solution in which DNA was prepared: 0.1 M NaCl in *a*; 0.25 M ammonium acetate, 0.5 mM EDTA, 10 mM sodium cacodylate, pH 7.0, in *b, d*; and 10 mM Tris-HCl buffer with 1 mM EDTA, pH 7.6, in *c, e*.

domains, thus supporting the hexagonal model and ruling out the possibility of a smectic phase (either smectic A or C).

Electron microscopy of freeze-fracture-etch replicas also supports the columnar hexagonal hypothesis. In the experiments we used glycerol at concentrations of up to 30% as it did not apparently alter the textures. Multiple fracture planes can be obtained but only three of the more significant are shown here (Fig. 3). One fracture direction reveals a superposition of layers constituted by molecules lying side-by-side in the layer plane (Fig. 3, fracture plane 1). Although the molecular orientation cannot be determined in the layers themselves, it can be on their edge, because some molecules are pulled out, whereas others remain in place during the fracturing process. This produces a 'fringe zone' in which molecules can be distinguished individually. The thickness of each layer is slightly larger than the diameter of the DNA molecule and the molecular orientation remains the same in the successive superimposed layers. Fracture planes nearly perpendicular to the molecular orientations are also seen (Fig. 3, fracture plane 2). Because of the unidirectional shadowing of the replica, only two directions out of three can be followed, separated by an angle of $\sim 60^\circ$. Fracture planes slightly oblique with respect to the molecular direction reveal one of the three main directions of the hexagonal lattice, which is underlined by the shadowing (Fig. 3, fracture plane 3). The observed periodicity is close to the distance separating the layers, from which the distance between molecules can be deduced. Values ranging from 3.5 to 4.0 nm were calculated from about ten measurements. These can be considered as a good estimation of the molecular interdistance. Textures and defects observed on freeze-fracture-etch replicas of this phase are also consistent with a columnar hexagonal phase and can be correlated with the patterns seen in the optical microscope (F.L., manuscript in preparation). Periodicities of ~ 50 nm would be expected in a smectic phase, but were never observed on the replicas.

Our microscopic results were confirmed by X-ray diffraction (performed at the LURE synchrotron radiation centre). The pattern given in Fig. 4 is typical of DNA oriented parallel to

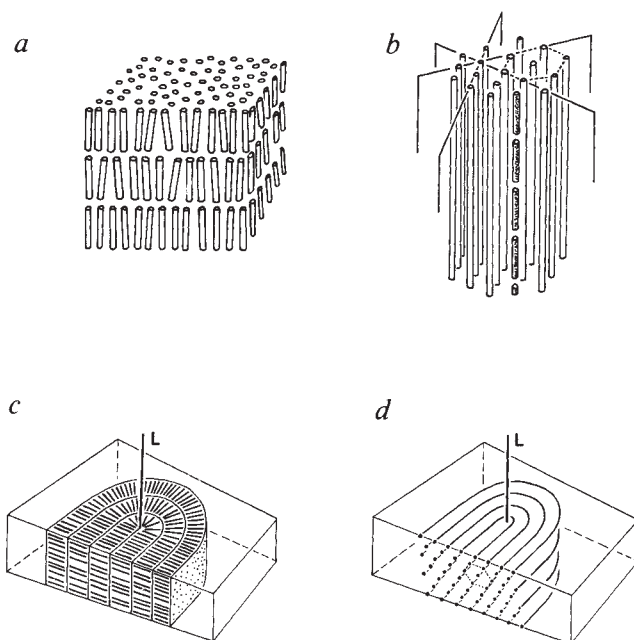
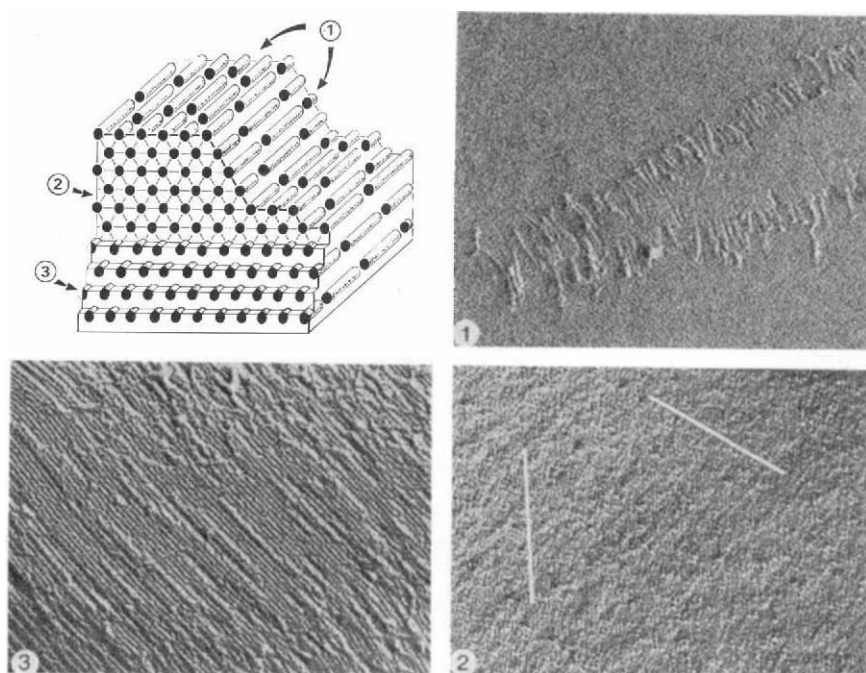


FIG. 2 Model of two liquid-crystalline phases in which a series of parallel layers can be defined. In a smectic phase (a) molecules are aligned and free to move in a series of parallel planes. The thickness of one layer corresponds to the length of the molecule in smectic A (molecules normal to the planes of the layers) or to a smaller value in smectic C (molecules tilted in the planes). In a columnar hexagonal phase (b) molecules are aligned along columns which draw a hexagonal network in section. There are three potential series of layers, according to the three main directions of the hexagonal network. In both structures, the layers are able to curve around disclination lines (L) but they lie parallel to the radius of the domain in a smectic phase (c) and normal to the radius of the domain in a columnar hexagonal phase (d).

FIG. 3 Freeze-fracture-etch electron microscopy of the highly concentrated liquid-crystalline phase of DNA. The drawing represents the organization of molecules in a columnar hexagonal phase. This model has three possible fracture planes: parallel to the layers in which molecules are aligned (1), perpendicular to the hexagonal network (2) and slightly oblique (3) which underlines one series of layers. These three situations are illustrated by micrographs labelled in the same way. 1, Fracture parallel to one series of layers in the lamellar structure of the hexagonal phase. One triangular-shaped layer, consisting of molecules lying in the plane and aligned side by side, is visible. The molecules can be followed individually at the edge of the layer. Their orientation remains the same in the successive superimposed layers. Magnification, $132,000\times$. 2, Fracture plane nearly perpendicular to the molecular direction. Only two directions of the hexagonal lattice can be seen because of the unidirectional shadowing. They are indicated by the white lines but their visualization remains difficult and needs some practice. Such patterns would also be expected in a smectic phase with a hexagonal lateral order of the molecules, but this hypothesis is ruled out by the analysis of the other fracture planes. Magnification, $70,000\times$. 3, Fracture slightly oblique. One series of layers is underlined by the shadowing. The measured periodicity is 4.0 nm. Magnification, $146,000\times$.

METHODS. Samples (in 0.25 M ammonium acetate, 10 mM sodium cacodylate, 0.5 mM EDTA, pH 7.0, with 30% glycerol; concentration ~ 250 mg ml $^{-1}$) were allowed to concentrate on gold discs to a thick consistency. (A drop of the same solution deposited onto a glass slide was allowed to dry in the same conditions and used as a control to check the



liquid-crystalline nature of the phase.) Samples were then quickly frozen in liquid Freon-22 and immediately transferred into liquid nitrogen. Fractures were made at -110°C under a 2×10^{-7} torr vacuum, etched at -100°C for 2–4 min, platinum-carbon shadowed at an angle of 45° and carbon-coated (Balzers BAF 400 T). After washing in distilled water, replicas were observed in a 201 Philips TEM at 60 kV accelerating voltage.

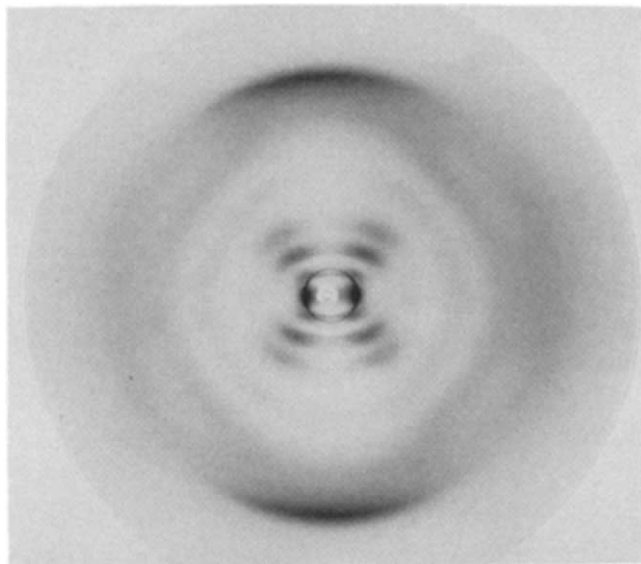


FIG. 4 Pattern of the highly concentrated DNA phase recorded on station D16 at LURE (Université Paris-Sud) using the synchrotron source at wavelength 0.1553 nm and a sample-film distance of 85 mm. The pattern is characteristic of DNA in the B form with a strong meridional arc at 0.333 nm (full width at half-maximum (FWHM) = $5 \times 10^{-2} \text{ nm}^{-1}$ and a double helix pitch of 3.33 nm). The strong equatorial reinforcement of the sharp arc (FWHM = $7 \times 10^{-3} \text{ nm}^{-1}$; reticular spacing 2.57 nm) reveals the hexagonal lateral order with interhelix distances of 2.97 nm. A DNA concentration of $\sim 380 \text{ mg ml}^{-1}$ can be estimated from the interhelix spacing. To prepare the sample, a 250 mg ml^{-1} solution of 50-nm DNA molecules in 0.25 M ammonium acetate, 0.5 mM EDTA, 10 mM sodium cacodylate, pH 7.0, was allowed to slowly evaporate, and flow-aligned in a quartz-capillary of 1 mm diameter.

the capillary axis. In the inner part, the intensity is located on layers corresponding to the helix pitch periodicity (3.33 nm). The first three layers above the equatorial layer are the three most intense, the fourth one is missing. In the outer part, a strong meridional arc located at 0.333 nm is observed. Such a pattern is characteristic of B-DNA chains with ten base pairs per helix turn, oriented perpendicular to the helix axis and separated, on average, by 0.333 nm (refs 15 and 16). A sharp arc with a strong equatorial reinforcement is also present. This arc reveals a long-range periodic lateral arrangement of the DNA chains which characterizes a hexagonal lattice of parameter length 2.97 nm. Higher order reflections of the hexagonal lattice are barely visible, revealing a significant displacement of the molecules around their average position ($\sim 0.15 \text{ nm}$). A more ordered structure is observed for higher concentrations and revealed by the appearance of several additional reflections which become visible when the interhelix spacing reaches 2.8 nm. This value can be compared with the data of Franklin and Gosling¹⁶ in which B-DNA fibres show interhelix spacings of 2.84 and 2.55 nm, attributed to the coexistence in the structure of two different hydration states. We never observed such a coexistence, although a progressive decrease of the interhelix spacing was apparent. This discrepancy could be attributed to the state of the specimen, which is mesophase rather than fibre. Complementary experiments, performed on samples whose textures were controlled optically, showed that the hexagonal parameter depends on the water concentration, with values ranging from 2.8 to 4.0 nm.

The longitudinal order of the DNA chains has been investigated with the SAXS station D24. The wavelength was 0.1608 nm and the sample-to-film distance was 1,850 mm. The film does not display any scattering maximum within the reticular range (75–7.0 nm), which thus excludes the possibility of a smectic-type layer arrangement.

The polyelectrolyte structure of DNA implies that water

surrounds the whole area of the cylinder and the decrease in interhelix spacing with increasing polymer concentration agrees with the hypothesis of a hexagonal phase. The structure remains liquid crystalline for molecular separations of up to $\sim 2.8 \text{ nm}$ (that is $\sim 0.6 \text{ nm}$ greater than the DNA helix diameter). Our results are consistent with previous X-ray experiments on DNA samples of different origin^{17,18}. In ψ -type DNA aggregates, which are short-range ordered structures, interhelix spacings were shown to be in the same range (2.92–4.38 nm)¹⁹. An ultimate degree of compaction of DNA is obtained when DNA is crystallized in the form of hexagonal platelets in which intermolecular distances are 2.76 nm (ref. 20) and 2.77 nm (ref. 21). Such structures present similarities with the dense packing of DNA that exists in virus capsids, where the DNA is also packed hexagonally with interdistances of 2.73–2.75 nm (ref. 22). However, in most biological structures the compaction of nucleic acids is not so great. In this case, the highly concentrated liquid-crystalline phase of DNA seems a more appropriate model than crystallized DNA to the understanding of *in vivo* DNA packing. For instance, the hexagonal order of the columnar phase can be compared with that in certain sperm heads^{17,18,23}. The value of such comparisons has already been demonstrated in the case of the cholesteric phase⁶. The hexagonal structure of the highly concentrated phase of 50 nm DNA molecules is consistent with previous results with longer DNA molecules¹¹ and suggests that the nature of this phase does not depend on the length of the molecules. We can assume that extremely long molecules, such as those found *in vivo*, behave in the same way. □

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ERRATUM

Multiscale periodic structure in the lo wake

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